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CONTENTS

Resistance to virus Y in the potato ..... R. H. Bagnall and R. H. E. Bradley 121

The "most probable number" method for estimating populations of plant pathogenic organisms in the soil ..... Otis C. Maloy and M. Alexander 126

Red stele root disease of the loganberry and strawberry caused by Phytophthora fragariae ..... W. E. McKeen 129

Host range, symptomatology, physical properties, and cross-protection studies of orchid virus isolates ..... Harry H. Murakishi 132

Serological and morphological relationships among orchid viruses ..... Harry H. Murakishi 137

Leaf streak of orchardgrass, timothy, and tall oatgrass incited by Scolecotrichum graminis ..... Samuel W. Braverman 141

Host nutrition in relation to Phytophthora root rot of avocado seedlings ..... F. T. Bingham, G. A. Zentmyer, and J. P. Martin 144

Snow mold of forage crops in Alaska and Yukon ..... J. B. LeBeau and Charles E. Logsdon 148

A method for studying absorption of streptomycin by using leaf disks of Sedum purpureum ..... John L. Lockwood 150

Relation of slime production to mechanism of wilting and pathogenicity of Pseudomonas solanacearum ..... Akhtar Husain and Arthur Kelman 155

Production and germination of oospores of Phytophthora infestans ..... J. J. Smoot, F. J. Gough, H. A. Lamey, J. J. Eichenmuller, and M. E. Gallegly 165

Growth inhibition as a mechanism in Dutch elm disease therapy ..... Carl H. Beckman 172

Phytopathological Notes:

The effect of water on the partial sterilization of barley seed by propylene oxide and by heat ..... L. E. Tyner 177

Internal uredial sori of Puccinia carthami with reversed polarity ..... M. L. Schuster 178

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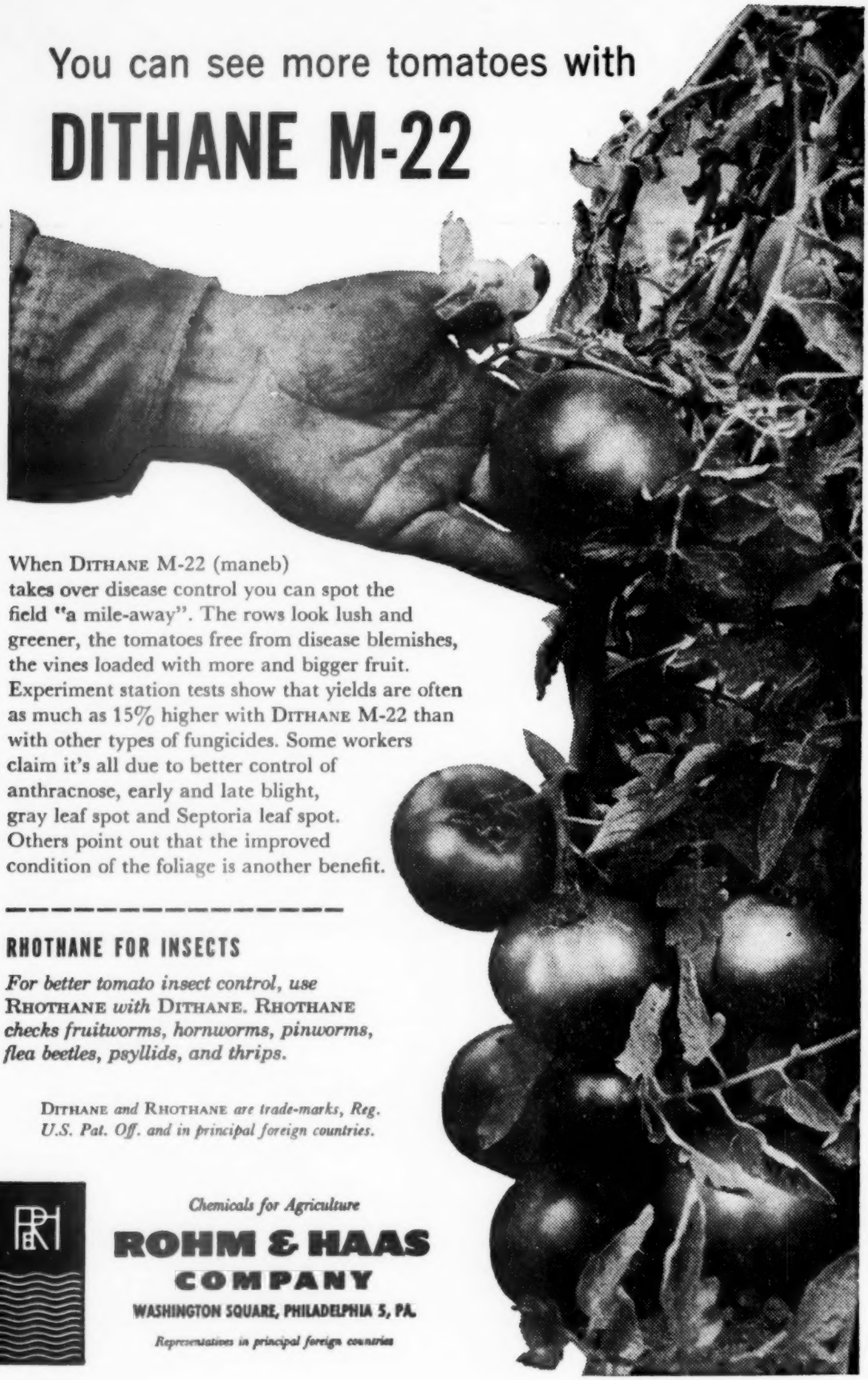

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RESISTANCE TO VIRUS Y IN THE POTATO [1]

R. H. Bagnall and R. H. E. Bradley [2]

SUMMARY

Potato varieties that reacted with necrosis to a common strain of virus Y were resistant in the field under conditions in which varieties that usually developed symptoms of mosaic were relatively susceptible. Further, when non-rogued stocks were grown in the field for 4 successive years, there was a progressive increase in the number of infected plants in varieties that developed mosaic symptoms, but the virus appeared to be self-eliminating in varieties that reacted with necrosis. This field resistance could not be reliably assessed by aphid inoculations with virus Y in the greenhouse. In greenhouse trials, infective aphids placed on young upper leaves transmitted the virus to more plants than did those placed on middle or lower leaves. Young upper leaves of infected plants were the best source of virus for aphids. Varieties with mosaic-type symptoms had a higher virus content than did varieties with necrotic-type symptoms; the former also were the better source of virus for aphids.

Resistance to virus Y in the potato has been reported by a number of workers (3, 5, 10, 18, 19, 20, 21, 23, 24) but there is no clear agreement as to its basis. Discussions have centered about resistance to initial infection (5, 8, 23), hypersensitivity (3, 11, 12, 13, 15, 17, 18), localization of the infection (9, 13, 16), and virus-inactivating mechanism (17). Some workers appear to regard these terms as essentially synonymous, whereas others consider them to be distinct in varying degrees. Nor is there agreement on the best way to test for resistance to virus Y. Such tests have been based on inoculation by tuber grafts, by stalk grafts, by various applications of infective sap, by artificial colonization with infective aphids, and by exposure in the field in proximity to infected plants. In nature, virus Y is spread by aphid vectors, notably the species *Myzus persicae* (Sulz.); hence, field testing is probably the most direct way to assess resistance that would be of practical worth, but to be reliable it must be repeated several years on a sufficiently large scale. Bawden and Kassanis (5) reported that susceptibility to virus Y in the field could be assessed from the results of aphid inoculations with virus Y in the greenhouse. Cockerham, M'Ghee, and MacArthur (11, 12), Hutton (15), and Bagnall (3) correlated resistance to virus Y with necrotic-type symptoms in infected plants. A method by which the resistance or susceptibility of potato seedlings to virus Y could be determined would be of value in a breeding program. With this in mind, we have studied the merits of some tests for resistance to virus Y.

Our results confirm the correlation between a necrotic-type reaction and field resistance, but plants that showed necrosis were not always resistant to aphid inoculation under glass. Therefore the results of our greenhouse tests did not as closely parallel the field results as did those of Bawden and Kassanis (5). Since several varieties proved highly resistant in both our field and our greenhouse tests, greenhouse testing may yet be of value in the development of a superior type of resistance.

FIELD TRIALS.—Three separate trials were made to evaluate the relative susceptibility of different varieties to infection with virus Y in the field. A new trial was commenced in each of the years 1951, 1952, and 1953, representing Experiments I, II, and III, respectively. Initially, in each experiment, replicated plots of healthy varieties were grown in proximity to Green Mountain potatoes infected with a common strain of virus Y. Each plot consisted of 50 plants in a 40-ft. length of a single row; there were 10 plots in a full row, with 3-ft. cross-alleys between plots, giving a total length of 427 ft. The second row, and every third row thereafter, was planted with virus-Y-infected Green Mountain, so that every plot was bordered on 1 side by diseased plants. In Experiments I and II, there were 10 varieties replicated 8 times, so that the layout consisted of 8 plot-rows and 4 diseased rows; in Experiment III, there were 12 varieties replicated 10 times, thus 12 plot-rows and 6 diseased rows. Somewhat different varieties were used in each experiment, but a balance was maintained between those reacting with necrotic-type symptoms and those reacting with mosaic symptoms. The plots in each experiment were rogued during the early part of the initial season to insure that any diseased progeny represented infection contracted during the experiment. There was no indication of first-year symptoms of virus Y infection in our field experiments.

At harvest, a sample of 50 tubers was collected from each plot, and the entire layout was replanted the following year, but with the rows of diseased plants absent. During the second year of each experiment, counts were made of diseased plants as soon as reliable symptoms were visible. These were taken to represent field spread of virus Y during the previous year's exposure.

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Experiment I was terminated after the 1952 readings and Experiment II in 1953. Experiment III, however, was continued to determine the cumulative effect of several years' exposure. Samples of 50 tubers from each plot were again harvested after the 1954 readings, and the layout was replanted in 1955 and again in 1956, the rows of diseased plants being absent. Infected plants were counted in each of these years, but none were rogued. A sample of 10 tubers, taken from each plot at the 1956 harvest, was planted in the greenhouse in the spring of 1957 for a final count.

In the field, virus-Y-infected plants of the varieties Epicure and Keswick and of Seedling F451 developed mild symptoms that were sometimes difficult to detect. As a check on the accuracy of field counts, tubers from selected plants were grown in the greenhouse during the late winter and early spring, under conditions especially favorable for symptom development.

From time to time, plants in the field and greenhouse were tested for virus Y by sap-inoculation on differential host plants. The veinal or "spot" necrosis reaction in Samsun tobacco (*Nicotiana tabacum* L.) simultaneously inoculated with viruses X and Y and the immunity of *Datura tatula* (L.) Torr. (4) were used as criteria.

Table 1 summarizes the results of Experiments I, II, and III. The symptom reaction of each variety or seedling is designated simply as mottle or necrosis, based upon second-year field symptoms, although both mottle and necrotic-type symptoms varied in severity. In general, tubers from necrotic-type reactors produced stunted plants, which had necrotic lesions on the stems and foliage; these died prematurely, sometimes shortly after emergence.

Table 1 shows that varieties reacting with mosaic symptoms were comparatively susceptible to field infection. Conversely, varieties reacting with necrotic-type symptoms were comparatively resistant. The varieties Craigs Defiance and Green Mountain appeared to be particularly susceptible, whereas Seedling F4519 and Kennebec had outstanding field resistance.

In Experiment III, the total number of infected plants in each of the mosaic-reacting varieties increased from year to year and approached 100 per cent in several, whereas in necrotic-type reactors there was little increase from year to year. Infected stocks of necrotic-reacting varieties appeared to be self-eliminating. This conclusion was confirmed in another way. Each year we planted diseased specimens of different varieties for comparison of symptoms. Stocks of mosaic-reacting varieties could be regrown indefinitely, but necrotic-type reactors, which produced stunted plants and small tubers, could be maintained for only 1 or 2 years. No tuber necrosis was seen, but eventually plants died without producing tubers.

Some degree of field resistance was shown by the mosaic-reacting varieties Canso and Irish Cobbler and Seedling F451. This was not comparable to the resistance of any of the necrotic-type reactors, but may be useful as a supplementary type of resistance.

GREENHOUSE TRIALS.—Since aphids spread virus Y in nature, aphid inoculations in the greenhouse presumably are the most likely to give results that apply to the field. The method of inoculating potatoes with virus Y in the greenhouse was similar to that which normally results in single aphids transmitting the virus to 50–70 per cent of young White Burley tobacco plants. Briefly, adult aphids or late-instar nymphs of *M. persicae* were kept without food a few hours, allowed 1 or 2 brief probes into a tobacco leaf showing well-developed symptoms of infection with virus Y, and then transferred to the plants to be inoculated, where they were allowed to feed several hours; afterwards the aphids were killed by fumigating the plants with nicotine (7).

Effect of plant age and inoculation site.—Before comparisons were made of the susceptibility of differ-

TABLE 1.—*Percentages of plants infected by virus Y when potato plants were grown 1 year adjacent to diseased plants (Experiments I, II, and III), and accumulative incidence of infection (Experiment III) when unrogued stocks were regrown 3 further years*

Variety	Expt. I[a] (1952)	Expt. II[a] (1953)	Experiment III				Reaction to infection
			1954[b]	1955[b]	1956[b]	1957[c]	
Craigs Defiance			31	51	95	100	Mosaic
Green Mountain	40	41	17	25	84	98	Mosaic
Keswick	17	20	7	18	79	92	Mosaic
Epicure	24	16	9	14	71	92	Mosaic
Irish Cobbler	26	17	8	7	44	73	Mosaic
Seedling F451[d]	22		6	8	41	66	Mosaic
Canso	15	5	10	10	43	73	Mosaic
Pontiac		3					Necrosis
Warba	8	0	1	1	8	3	Necrosis
Seedling 41956	5	1	1	1	3	2	Necrosis
Katahdin	6	0	1	1	6	6	Necrosis
Seedling F4519[d]	4		0.2	0	0.2	0	Necrosis
Kennebec		0	0	0	0.4	0	Necrosis

[a] Based on 400 tubers.

[b] Based on 500 tubers.

[c] Based on 100 tubers.

[d] Received from Mr. L. C. Young, Horticulturist, National Potato Breeding Project, Fredericton, New Brunswick.

ent varieties to aphid inoculation with virus Y, tests were made to determine the effect of plant age and inoculation site on the numbers of potatoes that became infected. Healthy plants of the variety Green Mountain were grown on a schedule that allowed simultaneous selection of plants 1, 2, 4, and 8 weeks after emergence. Aphids that had just probed infected tobacco were placed singly on an upper, middle, or lower leaf of plants of the different ages. The aphids were observed for at least an hour to ensure that they did not leave the leaf on which they were placed. Tests were done on 10 plants for each age and leaf position on 4 different occasions. When plants developed current-season symptoms at least 1 month before they died, such disease readings were taken as the numbers of infected plants; when current-season symptoms developed during the last month, however, 1 tuber was taken from each plant and regrown for second-year disease readings. The results (Table 2) show that except for 1-week-old plants, whose leaves differed little in age, more infections resulted from aphids placed on the upper leaves than from those placed on middle or lower leaves. Plant age had little effect on the numbers of infections so long as aphids were placed on the upper leaves, but when they were placed on middle or lower leaves, infections decreased as plant age increased. Thereafter, aphid inoculations were made by placing infective aphids on the young upper leaves of plants 2–4 weeks after emergence.

Varietal resistance to aphid inoculation.—Aphids that had just probed infected tobacco were transferred singly to young upper leaves of each of 10 plants of each of 11 varieties. Young healthy tobacco plants also were included to show whether the infectivity of the aphids was normal. The experiment was done on 5 occasions to make a total of 50 plants of each variety. The sharp distinction between the susceptibility of mosaic-reacting and necrotic-reacting varieties was no longer apparent under the conditions of this experiment (Table 3). Warba, resistant in the field, was among the most susceptible to aphid inoculation in the greenhouse, and the field-resistant seedling F4519 was rather susceptible in the greenhouse. Keswick, susceptible in the field, was the most resistant in the greenhouse. Green Mountain, susceptible in the field, appears in an intermediate position in the greenhouse tabulation. Katahdin, Kennebec, and Seedling41956, however, were relatively resistant under both conditions. Bawden and Kassanis (5) also reported that Katahdin is resistant in the field and to aphid inoculation under glass; our results are also similar to theirs in that King Edward was susceptible and Majestic moderately susceptible in the greenhouse.

One tuber from each plant in the foregoing experiment was planted for disease readings of second-year symptoms. For the most part, the results were the same as those based on first-year symptoms, but a notable exception was the fact that tubers from 5 of 6 Kennebec plants showing first-year symptoms produced healthy plants. First year symptoms in Kennebec usually consisted of a necrosis of the leaf inoculated by aphids. This suggests that the reaction in Kennebec resembles the local necrotic-type reaction rescribed by Hutton (16) and by Corbett (13).

In an experiment preliminary to the foregoing, 50 plants of each of the same varieties, excepting Kennebec, were colonized with 2, rather than with 1, infective aphids per plant. The differences in susceptibility were similar to those shown in Table 3; however, the level of infection was over 50 per cent, even in resistant varieties such as Katahdin and Keswick. Hence,

TABLE 2.—*Number of plants infected following aphid inoculation with virus Y of different leaves of Green Mountain potato plants at intervals after emergence*

Leaf inoculated	No. of plants infected (of 40 inoculated) when inoculation was made the indicated no. of weeks after emergence			
	1	2	4	8
Upper	15	18	20	16
Middle	18	9	6	9
Lower	10	8	7	6

TABLE 3.—*Varietal susceptibility to aphid inoculation with virus Y*

Potato variety	Reaction to infection[a]	Percentage of plants infected[b]
King Edward	M	52
Warba	N	44
Majestic	M	30
Seedling F4519	N	26
Green Mountain	M	22
Ontario	N	20
Canso	M	14
Kennebec	N	12
Katahdin	N	8
Seedling 41956	N	8
Keswick	M	6

[a] M = mosaic; N = necrosis.

[b] Based on 50 plants of each variety. In parallel tests with tobacco, 58 per cent of the plants became infected.

TABLE 4.—*Relative concentration of virus Y, as indicated by serological assays, in various hosts and its availability to aphids, as indicated by transmission tests to tobacco*

Virus source	Relative virus concentration[a]	No. of tobacco plants infected (of 50)
Tobacco	9.6	36
Potato		
Warba	5.2	3
Green Mountain	4.5	9
Seedling F451	4.0	12
Seedling 41956	2.4	4
Katahdin	2.4	3

[a] Expressed as reciprocal of highest dilution that gave a visible precipitate after 1 hour at 35°C.

resistant varieties also often become infected when the inoculation is severe.

Virus concentration and its availability to aphids.—More aphids became infective with virus Y when they probed the young upper leaves of infected plants than when they probed middle or lower leaves. In the experiment that demonstrated this, each aphid was observed until it probed once into an upper, middle, or lower leaf from a Green Mountain plant showing second-year symptoms of infection with virus Y. The relative infectivities of these aphids were then determined by placing them in pairs on young healthy tobacco plants. This was done several times with infected potato plants 4–9 weeks old and 24–36 in. high. A total of 160 pairs of aphids were tested for each of the 3 leaf positions. Those that had probed upper, middle, or lower leaves transmitted the virus to 78, 41, and 43, respectively, of the test plants.

Tests were made to determine whether varietal differences in the availability of virus Y to aphids are correlated with virus concentration measured serologically. Upper leaves were collected from field-grown plants showing second-year symptoms of infection. Pairs of aphids were observed until they each probed once into these leaves, after which they were transferred to young healthy tobacco plants. Ten pairs of aphids were tested for each variety on each occasion, and the experiment was done 5 times. Infected tobacco also was included as a source of virus. The numbers of test plants infected were used as a measure of the availability of virus Y to aphids. After aphids had finished probing these leaves, sap was extracted from the leaves and clarified by centrifuging 15 minutes at 10,000 G. This clarified sap was diluted with 0.85 per cent saline and 1-ml portions were titrated against equal volumes of suitably diluted virus Y antiserum. Virus concentration was taken as the inverse of the highest dilution of sap that gave a visible precipitate after the mixture was incubated 1 hour at 35°C. Tobacco was the best source of virus for aphids and it also had the highest virus concentration (Table 4). The best sources of virus in potatoes were the 2 mosaic reactors, namely Seedling F451 and Green Mountain; except for Warba, these also had the highest virus concentration. All 3 necrotic-type reactors were poor sources of virus for aphids, but Warba was again unusual in that it had the highest virus concentration of any of the potatoes.

DISCUSSION.—Our results show that a number of American potato varieties are field resistant to a strain of virus Y commonly found in eastern Canada; this resistance is correlated with necrotic-type response to infection. These varieties include the widely grown Katahdin, Kennebec, and Warba. Where such a variety is grown as a separate crop, resistance would be supplemented by the ease of roguing infected plants and possibly further by the fact that such plants would serve as poor sources of virus to aphid vectors. On the basis of necrotic-type reaction to virus Y, other varieties might be added to those mentioned above. These could include Canus, Cherokee, Chippewa, Earlaine, LaSalle, Marygold, Norkota, Saco, Sebago, Seneca, and White Cloud.

The relationship between the necrotic-type response and resistance is not yet apparent. Self-elimination of necrotic-type reactors is probably a factor in preventing year-to-year increases in the number of infected plants, but it does not appear to bear upon resistance to first-year infections. In the latter respect, certain mosaic-reacting varieties are more resistant than are others. This type of resistance may be similar to the initial resistance reported by Bawden and Kassanis (5) and by Cockerham, Grovier, and MacArthur (8). Cockerham et al suggest that such resistance would provide improved genetical background for the necrotic-type reaction with a resultant localization of most infections.

We have not attempted in the present work to classify necrotic-type reactions of the different varieties as "systemic," "lethal," or "localized," as did Hutton (16), although we recognize that these may be important considerations. All 3 reactions were observed on different occasions in each of several varieties. Nevertheless, we do not consider failure of a given reaction to meet some arbitrary definition of "hypersensitivity" (13, 17) to be grounds for rejecting it as a basis for field resistance.

It is true that the resistance we have found has been shown by ourselves and others (13, 19, 24) to be overcome to a greater or lesser degree under stress of severe inoculations or exposures. It is also possible that supplementary or better sources of resistance can be found among wild potato species (8, 23) and can eventually be bred into commercially acceptable varieties. Nevertheless, the type of resistance that has been correlated with necrotic-type response to virus Y is immediately available and has been shown to occur in some seedlings from most crosses (15, 18). Hence, necrotic-type reaction to virus Y should be favorably regarded in selection of new varieties. By contrast, no susceptible variety should be released if its field reaction is so mild as to make roguing difficult. This was encountered in some degree with several of the susceptible, mosaic-reacting varieties used in these experiments.

It seems advisable that determination of symptom reactions should be supplemented by field-exposure trials, at least until the basis of resistance to virus Y is better understood. Aphid inoculations in the greenhouse may help eventually to develop a superior type of resistance but do not appear to be a reliable guide to field resistance at the present time.

We have not considered, in the present paper, the possible effects of different strains of virus Y. Bawden and Kassanis (6), Corbett (13), Hutton (16), Darby, Larson, and Walker (14), Munro (22), and Ross (23) have reported different strains of virus Y. There may be differences in susceptibility of potatoes to different strains; a variety presumably would be more susceptible to a strain that induces a mosaic than to one that induces necrosis. For the most part, aphid-borne strains of virus Y do not appear to differ funda-

mentally in reactions incited in different potato varieties. Mild strains of virus Y have been reported by some of the above workers, but only Darby, Larson, and Walker (14) have described in any detail strains that cause only mosaic symptoms on such varieties as Katahdin, Kennebec, and Warba. Whether or not an increased use of resistant varieties will result in the appearance of new strains of virus Y not affected by the resistance in these varieties remains to be seen. It might be noted, however, that Katahdin has been grown widely for more than 20 years, and a great part of the American and Canadian crops are now planted with varieties that react with necrosis to our strain of virus Y.

In addition to the varieties noted, the virus-X-immune Seedling 41956 was found to be highly resistant to virus Y in both field and greenhouse tests. This should increase its value as a parent. Hutton (17) demonstrated linkage between the inheritance of resistance to virus Y and immunity from virus X. Indeed, the variety Saco, derived from Seedling 41956, is a necrotic-type reactor to our strain of virus Y; it is also termed immune from viruses A and X (1) and highly resistant to, if not immune from, virus S (2, 4).

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THE "MOST PROBABLE NUMBER" METHOD FOR ESTIMATING POPULATIONS OF PLANT PATHOGENIC ORGANISMS IN THE SOIL [1]

Otis C. Maloy and M. Alexander [2]

SUMMARY

The "most probable number" method has been adapted to the estimation of populations of 2 plant pathogenic fungi, *Fusarium solani* f. *phaseoli* and *Thielaviopsis basicola*, in the soil. The method requires the preparation of a dilution series of the soil sample and the inoculation of suitable test plants. After an adequate incubation period, the plants are examined for symptoms or signs of infection, and the number showing infection at the different dilutions is used to obtain the most probable number of organisms in the original inoculum. The normal soil microflora does not appear to interfere, even when the pathogen is present in low numbers in the test soil. Good agreement was observed between estimates of populations based on plate counts of the spore suspensions used to infest sterile soil and estimates by the "most probable number" method.

At the present time there is no satisfactory method of estimating the population of a plant pathogen in the soil. The plate count is not applicable because these organisms are generally poor competitors for nutrients on an agar medium and there are usually no media sufficiently selective for their isolation and enumeration. In addition, it is frequently very difficult to distinguish many pathogens from similar saprophytic species by laboratory tests. Plant pathologists occasionally infer populations on the basis of various disease indices (4, 7). These tests, however, are not necessarily estimations of the numbers of a specific microorganism except within certain narrow limits, such estimates being conditioned by the susceptibility of the host and the interrelationship of various environmental factors.

The procedure described in this paper has been used extensively for estimating bacterial populations in water and sewage (8) and has also been adapted to the enumeration of soil algae (1). In addition, Brockwell (2) has used indicator plants for the estimation of populations of nodule bacteria in peat cultures. The theory upon which the technique is based has been discussed in detail by Halvorson and Ziegler (6) and more recently by Cochran (5). The method consists of preparing a dilution series from the sample of liquid or soil and transferring aliquots from selected dilutions to replicate tubes of a suitable medium. The number of tubes in which there is growth or some specific biochemical change is recorded for each dilution, and the data for the highest dilution that resulted in growth in all tubes (or a suitably high proportion of positives) and for the 2 succeeding dilutions are used to form the code number. Thus, if 5 tubes received aliquots from each dilution and growth occurred in all tubes at 10^{-3}, in 3 tubes at 10^{-4}, in 1 tube at 10^{-5}, and in no tubes at higher dilutions, the code number would be 531. By use of a table of "Most Probable Numbers" (M.P.N.) such as that given Buchanan and Fulmer (3), the most probable number of organisms in the first dilution resulting in growth in all replicates, i.e. 10^{-3}, can be obtained. In this example, the value would be 11.0. Multiplication of this by the dilution factor gives 11,000, the population of organisms per unit of original inoculum. The same reference also gives tables for 2, 3, and 4 replicate tubes per dilution. Tables of "Most Probable Numbers" for 5 and 10 replicates are presented by Halvorson and Ziegler (6), who used the dilution corresponding to the second number of the code for calculation of probable numbers.

A series of experiments was carried out to determine if this method might give an estimate of the populations of plant pathogenic microorganisms in the soil.

MATERIALS AND METHODS.—Two fungi were studied as test organisms, *Fusarium solani* f. *phaseoli* (Burk.) Snyd. & Hans. and *Thielaviopsis basicola* (Berk. & Br.) Ferr. The host plants, used here essentially as testing "media," were Red Kidney bean plants (*Phaseolus vulgaris* L.) for the former microorganism and carrot (*Daucus carota* L. var. *sativa* DC.) disks (9) for the latter. In the experiments with the bean Fusarium, surface-sterilized bean seeds were planted in sterile soil contained in 3-in. pots, 1 seed per pot, and these were inoculated after germination and elongation of the hypocotyl.

A spore suspension of the test fungus was prepared and the spore concentration was determined by plate counts on potato-glucose agar acidified with 1 drop of 50 per cent lactic acid per 20 ml of medium and/or by direct microscopic count made with a hemocytometer. For the test, a quantity of this spore suspension was mixed with sterile soil to give a known number of spores per unit weight. A dilution series was then prepared with either sterile soil or sterile water as the diluent. Soil was used in the earlier experiments, but water was used subsequently because of the ease of making dilutions and in order to provide maximum opportunity for the inoculum to come in contact with the test plant. When the dilution series was prepared in soil, the soil of each dilution was divided into 5 equal portions and a bean seed planted in each aliquot or a carrot disk was placed on each. After

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TABLE 1.—*Estimates of populations of Fusarium solani f. phaseoli and Thielaviopsis basicola in infested soil made by the "most probable number" (M.P.N.) method and by plate count*

Expt. no.	Population per g soil	
	Plate count	M.P.N. method
	F. solani f. *phaseoli*	
1	92,000[a]	29,000
2	17,250	3,860
3	33,000	19,000
4	52,000	23,000
	T. basicola	
5	36,500	74,250
6	1,310[b]	1,973
7	1,310	1,393
8	1,310	1,333
9	1,310	860
10	1,310	1,600
11	62,000[a]	64,000
12	17,300[a]	11,500

[a] Direct microscopic counts used to measure the spore population.

[b] In this and the 4 following experiments, aliquots of the same spore suspension were used to infest all 5 soil samples.

a suitable incubation period, the test plants were examined for symptoms or signs of infection. When water was used as the diluent, 1-ml aliquots of the desired dilution were pipetted onto the test plants.

RESULTS.—Estimates of the pathogen population added to the soil were based on plate counts, with the exception of 3 experiments in 2 of which the plate method gave values considerably lower than those found by direct count. Both of these experiments involved *T. basicola*, and subsequent study indicated that the acid medium used for these plate counts suppressed the development of colonies.

In the experiments involving estimations of populations in infested soil, there was in general good agreement between the values calculated from plate or microscopic counts of the original spore suspension and those obtained by the M. P. N. method (Table 1). There is some indication that the M.P.N. procedure may tend to underestimate the number of viable units in the soil, a result not entirely unexpected, since conditions in the soil allowing for development of a single cell into an infective population are undoubtedly not as favorable as are those allowing for development of a colony from a single cell on an agar plate in the laboratory. Despite this possible difference, one that warrants further study, the M.P.N. values are a valid estimation of the numbers of pathogenic organisms added to the soil. In 1 study involving the addition of the same number of spores to 5 soil samples, the counts obtained varied by no more than plus or minus 1.5 times the soil inoculum.

TABLE 2.—*Estimates of populations of Thielaviopsis basicola made by the "most probable number" (M.P.N.) method in the presence of a competitive soil microflora.*

Ratio of infested soil to field soil	Population per g soil	
	Plate count	M.P.N. method
1:0	2,600	1,440
1:1	1,300	1,320
1:9	260	165
1:99	26	22
0:1		0

In order to determine if a population of a specific pathogen could be estimated when present in low numbers and when in competition with the normal soil microflora, the following experiment was carried out. Sterile soil was infested with *T. basicola*, the population being previously determined by plate counts. This infested soil was mixed in ratios of 1:1, 1:9, and 1:99 with a field soil believed to be free of *T. basicola*. Dilution series of the undiluted infested soil, the field soil, and the mixtures were used to inoculate carrot disks that had been soaked in a 0.05 per cent streptomycin solution for 16 hours prior to inoculation. The streptomycin treatment was included in order to prevent the development of bacterial soft rot, which tended to inhibit or obscure development of *T. basicola*. Positive growth was confirmed by microscopic examination of the carrot disks after incubation for 1 week.

The results indicate that the normal soil microflora does not interfere with the determination, since population estimates obtained when the infested soil comprised a very small proportion of the total volume of soil were in good agreement with those expected on the basis of plate counts (Table 2). It should be noted that numbers as low as 22/g can be determined by the procedure described. The results also show that a 100-fold variation in abundance of the fungus can be detected with no significant loss in accuracy.

Cochran (5) has stated that at 10-fold dilutions the factor for the 95 per cent confidence limit is 3.8 for 4 samples and 3.3 for 5 samples per dilution. Of 63 replicates in 16 separate experiments, the results of 79 per cent of the replicates and 94 per cent of the experiments were within the 95 per cent confidence limits of the number expected on the basis of plate or microscopic counts. In the 16 experiments reported, 3 to 5 replicates were used to estimate the mean count for the M.P.N. procedure, providing a total of 63 individual determinations. By the use of Cochran's values to establish the 95 per cent confidence limits for each of the counts and the means, it was found that the population estimates obtained by plating (or by microscopic count) fell within the confidence intervals for 15 of the 16 experimental M.P.N. means and for 50 of the 63 individual determinations.

DISCUSSION.—The "most probable number" method may be applied to the estimation of populations of plant pathogens when one considers that the host plant actually serves as a selective medium, that infection and subsequent symptom expression indicates the presence of the pathogen, and that an environment conducive to infection generally can be created and maintained. An assumption that must necessarily be

made is that a single microbial unit (spore, mycelial fragment, sclerotium, etc.) can cause infection following growth and development. It is possible, however, to express the results in terms of "infection units" in order to circumvent this assumption.

The results obtained with infested soil, both in the presence and absence of a competing microflora, were generally well within the statistical limits, although in a number of experiments the estimates were somewhat lower than would be expected on the basis of the plate counts. It should be recognized, however, that the 3 methods mentioned here for enumerating the fungi actually determine different populations. The direct microscopic count determines the total number of propagative units present, the plate count gives the number of viable units, whereas the "most probable number" method estimates the infective population. It is not unreasonable, therefore, to expect that each method might give a somewhat lower value than the preceding one.

The incubation period is an important factor to be considered when the "most probable number" method is used, just as it is in the plate count. In the one experiment that gave results falling outside of the 95 per cent confidence limits (Experiment 2) the fungus was *F. solani* f. *phaseoli* and the observation was made after only 4 weeks' incubation. It is probable that not enough time was allowed for adequate symptom development and that this resulted in the low estimates. In all other experiments involving this fungus, the incubation period was extended to 6 weeks. Another possibility is that the infective population of the culture comprised only a small proportion of the viable population.

This method conceivably may be applied to any soil-borne pathogen provided that a suitable test plant is available and that infection by that organism can be recognized by appropriate symptoms or signs. In those experiments involving the use of carrot disks for the determination of *T. basicola*, it was observed that soft rot occasionally occurred. It is likely that an appropriate modification of the procedure might be suitable for determining populations of such soft rot bacteria. Undoubtedly an extensive list could be compiled of organisms to which this method might be applied, including fungi, bacteria, and plant parasitic nematodes. The method described provides a valuable tool in several plant pathological problems. It should be of value in ecological studies involving plant pathogens in the soil, since it offers a means of determining the relative abundance of specific organisms whose estimation would otherwise be impossible. In studies concerned with the control of plant diseases, the M.P.N. technique may also prove useful in ascertaining the effects of various control measures on pathogen populations.

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RED STELE ROOT DISEASE OF THE LOGANBERRY AND STRAWBERRY CAUSED BY PHYTOPHTHORA FRAGARIAE [1]

W. E. McKeen [2]

SUMMARY

On Vancouver Island an unthriftiness of most loganberry plantations has resulted from necrosis of loganberry roots during autumn and winter when the soil is cool and very wet. The root decay is incited by a *Phytophthora* sp. that invariably is very closely followed by *Pythium* spp. Difficulty is experienced in isolating the primary pathogen because of its close association with *Pythium* spp., its relatively slow rate of growth, and its inability to grow at high temperatures and on certain media. An organic substance in soil water apparently stimulates the production of sporangia. This pathogen is considered a strain of *Phytophthora fragariae* because of similarity in morphology, cultural habits, growth-temperature relations, and capability of causing red stele in the Huxley variety of strawberry and in *Fragaria vesca*.

The relationship of the loganberry race of *P. fragariae* to the race that causes root degeneration of raspberry is unknown because the latter has not been obtained in pure culture.

A general decline has been observed in vigour and yield in many loganberry plantations on Vancouver Island (4) and in some raspberry plantations of the Fraser Valley of British Columbia. The most noticeable symptoms of decline are unthriftiness and, occasionally, death of plants. Although cane gall and crown gall were found to be very serious in many plantings, it was apparent that the tumor-producing bacteria could not be responsible for this serious deterioration, because some plants free from galls were very unthrifty.

An examination of the roots during the winter season showed that a great many were necrotic and frequently were completely decayed. Because of this, a study was undertaken to determine the cause and nature of the root degeneration. Although raspberry is parasitized by the same or very similar pathogens as is loganberry, the latter was used in this investigation because it produces large white roots on its "tips" throughout the fall and winter season. The ends of loganberry canes produce roots in the fall and shoots in the spring; these young propagating plants are called "tips." These roots are excellent for use in etiological work.

SYMPTOMS.—Loganberry plants appear to develop normally during early spring, but close examination shows that fewer new shoots emerge from the old crown than is normal, particularly in the low-lying areas of the field. Sometimes new shoots begin to emerge from the soil, but suddenly growth stops and they become brown and necrotic. Later in the season, when the fruit is beginning to mature, a physiological drought and starvation occur. The leaves become dry, brittle, curled, and frequently discoloured. The first berries to ripen may be fairly well developed, but the main crop is of inferior quality. The later berries never mature properly but dry and turn black on the receptacle.

When the unhealthy plants are removed in midsummer, the crown and root system is not necrotic and new white healthy roots are present. The root system is restricted to a relatively small area about the crown (Fig. 2) of the plant, however, and does not appear to be large enough to support the aboveground parts during the fruit-maturing period when the soil is dry.

Soon after the "tips" have rooted, infection begins to appear at the extreme tip of the root (Fig. 1), and the prevalence of infection increases as the winter progresses. The tip of the root first appears water-soaked and then turns chocolate brown, and this discolouration continues to advance up the stem. A reddish brown discolouration of the stele occurs in advance of the cortical browning (Fig. 3).

ISOLATION OF CAUSAL ORGANISM.—Only "tip" roots were used, since the crown roots are unsuitable because of their dark colour, their frequent superficial infestation with many saprophytic and weakly parasitic organisms as well as with primary parasites, and the small size of the young white crown roots. Also the "tip" roots are free from laterals and grow vigorously during the infection period.

From pieces of infected roots placed in water, sporangiophores and sporangia developed in about 24 hours; the presence of Phytophthora was easily ascertained, and this encouraged the author to continue attempting various techniques in isolation. The early failures of isolation resulted from various causes, such as high room temperature, unsuitable media, and contamination with bacteria and with *Pythium* spp. It frequently required 2 or 3 days after the root was placed in media for hyphae to emerge from the infected root. In potato-dextrose agar, the mycelium might become ¼ in. in length before cessation of growth. A day later the protoplasm within the hyphae would be irregularly broken into segments, and it was obvious that it was dead. This effect was brought about by dextrose (6).

Success in isolation was achieved when a very short section of the stele, in which the transition in colour occurred, was embedded in water agar or in oatmeal agar as it was about to gel. These isolations were

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The investigational work was completed at the Plant Pathology Laboratory, Saanichton, B. C.

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placed at a temperature of about 15°C.

PROOF OF PATHOGENICITY.—Steam-sterilized soil that was artificially infested with Phytophthora cultures grown on agar and in oatmeal-sand medium was placed in trays in Wisconsin temperature tanks maintained at 5° and 10°C. Both loganberry "tips" and seedlings were planted in infested and steam-sterilized soil. A very marked difference was observed in the growth of the seedlings (Fig. 4), and the roots of the "tips" soon became necrotic. It was also found that the Cascade berry was equally susceptible.

The British Sovereign strawberry, red garden beet, pea, corn, tomato, turnip, cabbage, radish, sweet pepper, spinach, lettuce, aster, carnation, poppy, nasturtium, zinnia, hollyhock, marigold, Canterbury bell, Kochia, cynoglossom, verbena, campanula, phlox, and petunia remained free of disease. The fungus was unable to grow also in potato tubers, apples, and green and ripe tomato fruit.

It was found, however, that the Huxley variety of strawberry and *Fragaria vesca* were susceptible.

LIFE HISTORY OF CAUSAL ORGANISM.—Zoospores that have encysted at the root cap send out a germ tube that penetrates the cortex. The hyphae grow in the stele, which turns reddish in colour, until they almost reach the crown. Asexual reproduction is carried on while the root is parasitized if moisture and temperature are suitable. If infected roots are immersed in water, sporangiophores immediately begin to radiate from the infected tissue.

When cultures of Phytophthora grown on oatmeal and lima bean agar are placed in distilled water, only a very limited number of sporangia are produced. Numerous sporangia are formed, however, if water from soil drains or water filtered through soil is added to these cultures. Soil water sterilized in an autoclave

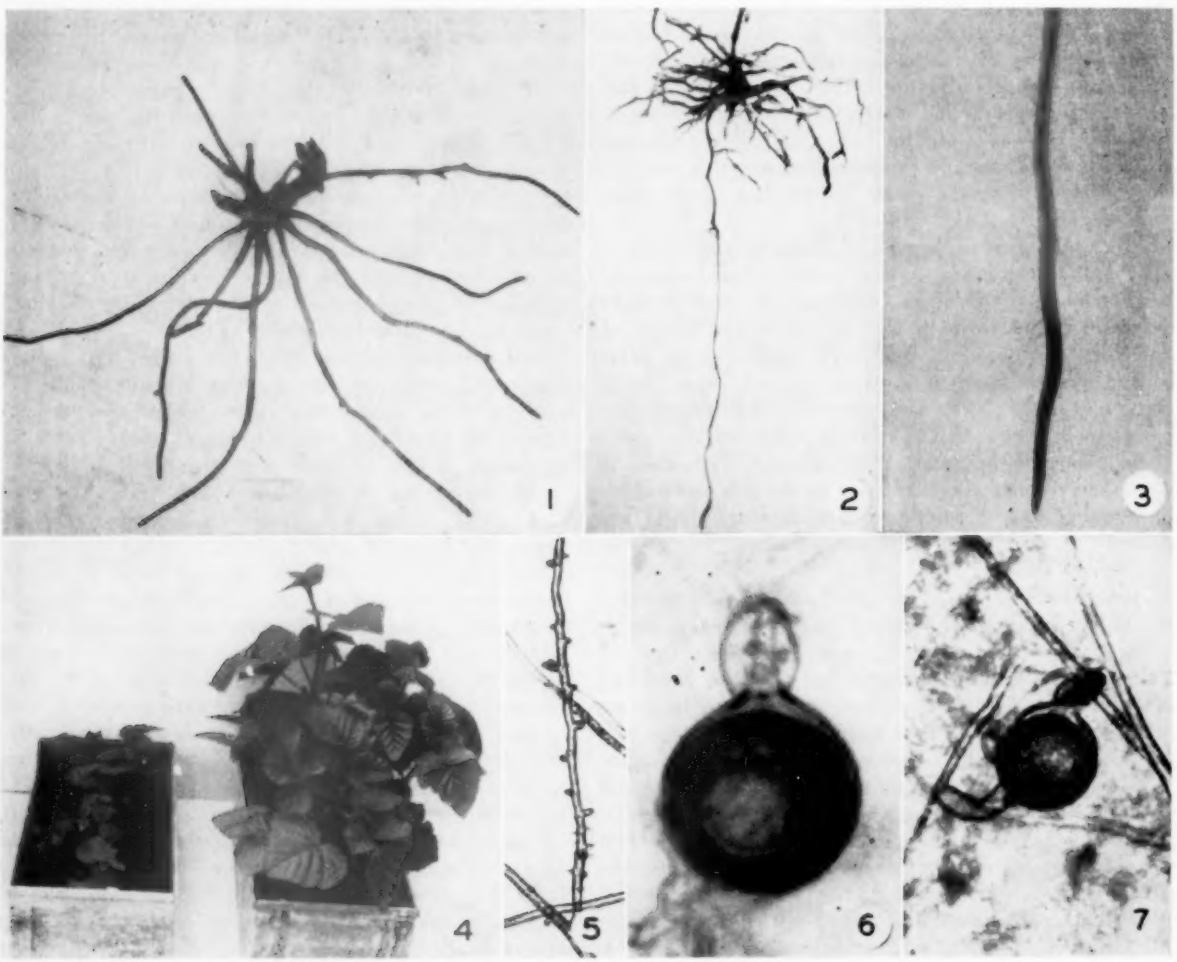

FIG. 1-7. Fig. 1. A young root system of a loganberry "tip" infected by *Phytophthora fragariae*. Observe that the tips of the roots are black and necrotic.—FIG. 2. An older "tip" root system. Most of the roots are restricted to a relatively small zone about the crown.—FIG. 3. A red stele in an infected root. The red stele extends into the white cortical tissue.—FIG. 4. Loganberry plants that were grown at 10°C. The soil was steam-sterilized prior to planting and Phytophthora inoculum was added to the tray at the left.—FIG. 5. A hypha with short lateral protuberances.—FIG. 6. An oogonium with an amphigynous antheridium.—FIG. 7. An oogonium with 2 paragynous antheridia.

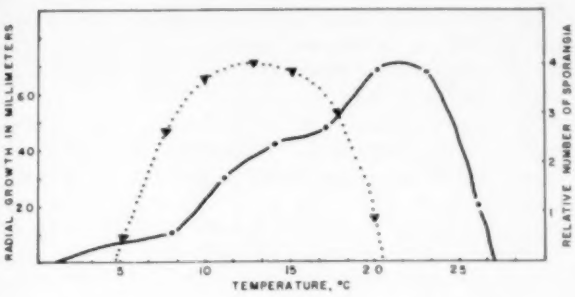

FIG. 8. Influence of temperature on mycelial development (————) and sporangial development (. . . . .) of *Phytophthora fragariae*.

is equally as effective as soil water sterilized in a Seitz filter; however, when soil water is evaporated to dryness and flamed at a temperature under 400°C until it is a white ash, the ash (dissolved in aqua regia, diluted to the original volume, and adjusted to pH 7, the original pH) is not effective in stimulating the production of sporangia. The maximum number of sporangia is produced at a temperature of 12°–17°C. This is about 10° lower than that required for maximum radial growth (Fig. 8). If the bathing solution is left at a low temperature, liberation of zoospores is very gradual but becomes very rapid if the temperature is elevated a few degrees. Zoospores encyst immediately at 30°; after 10 minutes at 28°, the majority are encysted. At 25° and lower, they remain active for about 5 hours, even in water containing ice and snow.

The zoosporangia are borne terminally on undifferentiated sporophores that average about 90 μ but may vary in length from 10 to 270 μ. The zoosporangia, which soon become numerous, are at first spherical but later become pear shaped. They are nonpapillate and measure about 30–74 $\mu \times$ 26–52 μ, with an average of 55 $\times$ 39μ. Their wall is hyaline and slightly thickened at the distal end but otherwise thin. Germination usually occurs by the liberation of about 40 zoospores following the disappearance of the slightly thickened apical wall. Prior to the liberation of the zoospores, the protoplasm within the sporangium is broken into rounded bodies with 2 flagella; however, no movement of the zoospores occurs until they flow out of the sporangium.

After the evacuation of a zoosporangium, a new one may develop from within the base of the original empty sporangium; the new sporangium may remain within or protrude beyond the original, depending on the length of its sporangiophore. Such sporangial proliferation may be repeated, 5 times being the maximum observed.

The zoospores are ellipsoidal in shape, each having a definite groove on 1 anterior side. They may be observed most easily in water placed in ground concave depressions of transparent glass spot-plates. The particular dark field (not the normal dark field) obtained with the Leitz phase-contrast microscope proved most suitable for observing the zoospores and their movements. The flagella appear as white lines.

The zoospores are about 13 μ in length, and they have an anterior flagellum, about 20 μ in length, that points straight forward at all times and never waves about. In the mounts examined, the posterior flagellum, about 50 μ in length, was always trailing in a waving fashion directly behind the zoospore, which never stopped swimming until it encysted. The zoospore appeared to rotate as it moved forward and changed direction by flipping its posterior end toward its concave side and thus turned in the direction of its concave side. Two zoospores that were attached just behind their body by their posterior flagella were capable of making vigorous jerks in all directions until they separated.

At room temperature, the zoospores usually swam for 3–4 hours before they began to encyst. If they were sealed in a drop of water under a cover slip by paraffin wax, however, they encysted within 3 minutes after sealing. This may be due to haptotropism. First indications of encystment were a decrease in swimming speed and a rounding of the protoplast. As swimming stopped, the flagella began to lash from the point of insertion in an uncontrolled fashion. Usually the flagella wrapped about the protoplast and were no longer distinguishable. Immediately after encystment, a germ tube was produced that grew about 32 μ per hour at 20°C and sometimes became 1 mm in length in distilled water.

Sexual organs are formed in large numbers in the central cylinder of the diseased roots grown in infested soil. Soon after the fungus is isolated and cultured on oatmeal agar, sexual organs are formed but their ability to do this is lost during culturing.

The oogonia (Fig. 6, 7) are terminal or lateral on very short stalks. They vary from 31 to 53 μ in diameter (average 41 μ) and possess a thick smooth wall. The oospores, 26–40 μ in diameter, average 33 μ, and are spherical and lie free in their oogonia. Their wall is smooth and hyaline and averages 2 μ in thickness. They contain finely granular protoplasm in which a single large oil globule is embedded. The heating of infested soil for 2 days at 30°C or higher killed the oospores; susceptible plants did not become diseased when they were planted in the heat-treated soil.

In pure culture, the mycelium is fairly uniform in diameter and averages about 6 μ in diameter, but the range is from 4 to 9 μ. Fingerlike protuberances are produced if the medium is not suitable for optimum growth (Fig. 5). The fungus will not grow on media to which has been added reducing carbohydrates (6).

DISCUSSION.—In the past, a very limited amount of investigation has been carried out on the disorders of the underground parts of cane fruits (5, 2). This has been due to great difficulty in observing and investigating the regeneration that occurs on a dark-coloured perennial root system. Also, a great many of the primary infections begin during the winter period, and aboveground symptoms usually appear after a con-

siderable lapse of time; consequently the trouble is not properly diagnosed, and studies are not undertaken at the appropriate time of the year.

The fact that the Phytophthora causing loganberry root degeneration is a race of *P. fragariae* is of considerable importance (1, 3). Not only does it show that there is more than 1 race of *P. fragariae* but also that it might be very unwise to rotate loganberries and strawberries. Furthermore, the complexity of breeding strawberries resistant to red stele is enhanced.

Dryness, rather than high temperature, is the first limiting factor to express itself in the spring. The sexual stage serves to oversummer rather than to overwinter this pathogen.

Because the ash from soil water is not effective in inciting the production of sporangia and zoospores, some organic substance in soil water must be stimulatory.

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HOST RANGE, SYMPTOMATOLOGY, PHYSICAL PROPERTIES, AND CROSS-PROTECTION STUDIES OF ORCHID VIRUS ISOLATES [1]

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SUMMARY

Virus isolates selected from diverse genera of the *Orchidaceae* caused a wide range of symptoms in the source plants. In comparative studies made with the isolates, however, the symptomatology, host range, and physical properties were, in the main, remarkably similar. In most cases, symptoms produced by the several isolates on 7 genera of orchids and on *Datura stramonium* L. were identical. Two new local-lesion hosts in the *Orchidaceae*, *Miltonia bluntii* Reichb. f. and *Arundina bambusifolia* Lindl., were susceptible to all isolates. *Vanilla planifolia* Andr. was systemically infected by 4 of the isolates tested but exhibited no distinct symptom. The isolates also caused no symptoms in plants of 23 nonorchidaceous genera, but recovery tests to *Spathoglottis plicata* Bl. indicated that virus multiplication had occurred in *Cucumis sativus* L., *Nasturtium majus* L., *Zinnia elegans* Jacq., *Passiflora edulis* Sims., and *Oryza sativa* L. The physical properties of 4 of the isolates, as determined by tests on *Spathoglottis plicata*, were similar to those described for Cymbidium mosaic virus. This basic uniformity in host reactions is believed due to the presence of Cymbidium mosaic virus or strains of this virus in all of the isolates.

In some cases, however, differences in behavior were noted. These were rather striking and distinctive. An isolate from Cattleya induced a top necrosis of Spathoglottis and a flower color break of Cattleya. An isolate from Dendrobium caused distinctive local lesions in Cymbidium and a color break and mosaic of *Dendrobium superbum* Reichb. f. Similarly, Vanda mosaic virus was the only isolate to cause a color break in Vanda flowers. In cross-protection tests, it was demonstrated that the isolate which causes Cattleya color-break contains a second virus unrelated to Cymbidium mosaic virus. A mixture was also suspected in isolates of Dendrobium mosaic virus and of Vanda mosaic virus but was not demonstrated in this study. Cattleya color-break virus proved to be the most stable of the isolates. It was not inactivated *in vitro* at 80°C for 10 minutes nor when stored at room temperature (20–30°C) for 32 days. Properties of Vanda mosaic virus, determined in an earlier test, indicated that this virus is less stable than the other isolates. It was inactivated at 60°C and after aging for 5 days.

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Diseases caused by juice-transmissible viruses have been reported in plants of many genera of the *Orchidaceae* (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13). These genera include *Cattleya*, *Cymbidium*, *Dendrobium*, *Grammatophyllum*, *Laelia*, *Miltonia*, *Oncidium*, *Odontioda*, *Odontoglossum*, *Spathoglottis*, *Vanda*, and *Zygopetalum*. Very little information is available, however, on the transmissibility of any virus from 1 orchid genus to another, although species of both *Cymbidium* (3, 4) and *Spathoglottis* (9) have been shown to be susceptible to viruses isolated from other genera of orchids. This aspect of virus transmission among different generic groups of the *Orchidaceae* is of considerable concern to growers, since many of them propagate clonally and maintain in a single nursery hundreds of orchid plants representing several genera. Furthermore, accidental transmission of orchid virus with contaminated knives and shears has been demonstrated to be readily accomplished under experimental conditions (4, 5). It was therefore of interest to determine, with the isolates and plants available in Hawaii, the host range, symptomatology, stability, and interrelationships of viruses associated with distinctive symptoms in plants of diverse genera of orchids. A brief abstract of preliminary findings has been published (10).

MATERIALS AND METHODS.—*Virus cultures and test plants.*—Source plants, representing 5 genera of orchids, were obtained from growers in the Hawaiian Islands and were selected for their distinctly different symptoms. Cattleya necrosis virus (CNV) was isolated from a Cattleya hybrid with necrotic leaf streaks. Cattleya color-break virus (CBV) was obtained from a Cattleya-Brassavola hybrid that had leaf mosaic and flower color break. Cymbidium mosaic virus (CMV) was isolated from an unnamed Cymbidium hybrid with leaf mosaic. Dendrobium mosaic virus (HMV) was maintained in *Dendrobium superbum* Reichb. f., locally known as Honohono orchid, that had leaf mosaic and flower color break. Spathoglottis diamond spot virus (SDV) was isolated from a hybrid of *Spathoglottis plicata* Bl. and *S. vanoverberghii* that had systemic ringspot symptoms. Vanda mosaic virus (VMV) was maintained in Vanda Miss Joaquim that displayed leaf mosaic and blossom color break. Each of the isolates and subinoculated plants were kept on separate benches in an orchid lath house, and cultural operations were reduced to a minimum to prevent accidental transmission. To prevent infestations by insects, the plants were sprayed periodically with insecticides. Test plants such as species of *Cattleya*, *Cymbidium*, and *Spathoglottis* were seedlings that were free of any disease symptoms. Species of *Miltonia*, *Vanda*, *Dendrobium*, and *Vanilla* were propagated from healthy divisions or cuttings. All plants were maintained in a rapidly growing condition, since preliminary trials indicated that only young succulent tissue could be consistently infected.

Method of inoculation.—Frozen, weighed leaf samples were thawed and ground in a mortar to which distilled water was added at a ratio of 2 ml water to 1 g of tissue. The crude juice was strained through cheesecloth and applied with a cotton swab to leaves previously dusted with fine Carborundum. After the leaves were rinsed with water, the plants were placed on benches in the lath house.

ORCHID HOST RANGE AND SYMPTOMATOLOGY.—Symptoms caused by the isolates were in most cases remarkably similar, but some outstanding differences were noted. Their effects on plants of 8 genera of orchids are given below. From time to time, the supply of orchid plants was limited so that in some cases recovery tests were not attempted.

Cattleya hybrids.—With the exception of VMV, all isolates caused leaf and flower sheath necrosis about 5 weeks after inoculation. This was followed a few days later by necrotic sunken streaks and ringspots on the inoculated leaf (Fig. 1,A). After this stage, 6 weeks or more after inoculation, the necrosed leaf was cast, leaving only the blackened pseudobulb. Unless virus infection was followed by soft rot bacteria, the plant was not ordinarily killed, but new leaf growth was stunted, cupped, brittle, and often pock-marked. On CBV-infected plants, a coarse, white color-break of the flowers developed and was accompanied by a severe leaf mosaic. HMV induced a slight color break of the flowers and purple streaks on the new growth on 2 of 5 plants inoculated. None of the other isolates caused flower symptoms in Cattleya plants. Of 7 plants inoculated with VMV, only 1 reacted with a mild mottle. In previous experience with this isolate (5), no infection was obtained in Cattleya. Transfers back to the original host genus were successful for all isolates except VMV.

Cymbidium hybrids.—On Cymbidium hybrids, all isolates caused varying degrees of leaf mosaic; however, HMV was the only isolate that induced local, necrotic, diamond-shaped lesions. These appeared within 4 weeks after inoculation and were followed by a severe chlorotic mottle of the new growth. CBV-infected plants developed a coarse mottle 4–8 weeks after inoculation. All plants except those inoculated with VMV later developed the black streaks and necrotic patterns characteristic of Cymbidium mosaic (1). VMV caused only a mild leaf mosaic. Recovery tests from the Cymbidium plants with mosaic to the originating host genera were successful with CNV, CMV, and SDV, but not VMV and HMV. Attempts to recover CBV on Cattleya sometimes resulted in only partial reproduction of symptoms. Leaf necrosis was consistently reproduced, but this was not accompanied by the usual floral symptoms in some cases.

Spathoglottis plicata.—All isolates except VMV caused diamond-shaped, necrotic, local lesions 2–4 weeks after inoculation. The symptoms were similar to those previously described on this host (6). On the new growth, all isolates except CBV caused a faint to mild mosaic. CBV-inoculated plants developed a severe, coarse leaf mosaic. Six to 8 weeks after inoculation, all isolates induced systemic ringspot symptoms. The concentric ring patterns (Fig. 1,E) were in contrast to the solid, necrotic, local lesions and extended

into the leaf base areas as partial rings and irregular necrotic markings. In addition to these symptoms, CBV caused a distinctive top necrosis, resulting in a blackened center shoot (Fig. 1,D). In recovery tests to the originating host genera, VMV and HMV failed to reproduce symptoms but other isolates did so consistently.

Arundina bambusifolia Lindl.—In plants of this species, all isolates caused local, necrotic streaks that were followed by a yellowish, mosaic mottle in 6–8 weeks (Fig. 1,C). Recovery tests were attempted with isolates CMV and SDV only. In both cases, original

FIG. 1. Leaf symptoms caused by orchid virus isolates. A) Local necrotic streaks and ringspots in Cattleya hybrid. B) Local lesions in *Miltonia bluntii*. C) Necrotic streaks and mosaic in *Arundina bambusifolia*. D) Top necrosis of *Spathoglottis plicata*. E) systemic ringspot in *S. plicata*.

symptoms were reproduced on plants of *Cymbidium* and *Spathoglottis*, respectively.

Miltonia bluntii Reichb. f.—All isolates produced solid, reddish to reddish-brown local lesions in 10–14 days (Fig. 1,B). The spots were elliptical and averaged 1–2 × 2–6 mm. Later, leaves of infected plants developed a mosaic, and the plants were noticeably stunted. Transfer of the virus from Miltonia back to the original host was accomplished with all isolates except VMV.

Vanda Miss Joaquim.—Only isolates VMV and CBV caused symptoms in this host. VMV caused a leaf mottle and blossom color break whereas CBV induced mild leaf mosaic only.

Dendrobium superbum.—HMV, originally isolated from Dendrobium, was the only isolate that caused symptoms on this host. Mosaic symptoms required from 1 to 3 months, whereas the incubation period for expression of color-break symptoms varied from 3 months to 1 year.

Vanilla planifolia Andr.—Four of the isolates, CNV, CMV, HMV, and SDV, were tested on this host. They produced no noticeable symptom except a slight downcurving of the tips of the new leaf growth; however, successful reisolations were made to Spathoglottis in each case.

NONORCHID HOST-RANGE STUDIES.—Plants representing 15 nonorchid families and 23 genera were grown from seed and inoculated when they were at the cotyledonary to the 2-leaf stage, but no symptoms could be detected. These included the following AMARANTHACEAE—*Gomphrena globosa* L.; APOCYNACEAE—*Vinca minor* L.; BALSAMINACEAE—*Impatiens balsamina* L.; CARICACEAE—*Carica papaya* L.; CARYOPHYLLACEAE—*Dianthus carophyllus* L.; COMPOSITAE—*Centaurea cyanus* L., *Cosmos bipinnatus* Cav., *Lactuca sativa* L., *Zinnia elegans* Jacq.; CRUCIFERAE—*Brassica napus* var. *chinensis* L., *Raphanus sativus* L.; CUCURBITACEAE—*Cucumis sativus* L.; GRAMINEAE—*Oryza sativa* L., *Zea mays* L.; LEGUMINOSEAE—*Lathyrus odoratus* L., *Phaseolus vulgaris* L.; MALVACEAE—*Althaea rosea* Cav.; PASSIFLORACEAE—*Passiflora edulis* Sims.; SOLANACEAE—*Capsicum frutescens* L., *Lycopersicon esculentum* Mill., *Nicotiana tabacum* L. var. Turkish; TROPAEOLACEAE—*Nasturtium majus* L.; UMBELLIFEREAE—*Apium graveolens* L. In plants of *Datura stramonium* L., however, all isolates produced the characteristic local-lesion symptoms caused by Cymbidium mosaic virus (4, 13). Recovery tests from some of the symptomless test plants were made by taking a composite sample of only the new leaf growth 4–6 weeks after inoculation and applying the extracted juice to healthy Spathoglottis seedlings. Virus recovery was successful with the following symptomless hosts and isolates: *Cucumis sativus* (SDV), *Nasturtium majus* (CNV, CBV, CMV, SDV, and VMV), *Oryza sativa* (CNV and CMV), *Passiflora edulis* (CNV, CMV, and HMV), and *Zinnia elegans* (CBV and CMV).

TABLE 1.—*Number of test plants[a] infected following rubbing with heated (for ten minutes), diluted, or aged juice from orchid plants infected by various Hawaiian virus isolates[b]*

°C	CNV	CBV	CMV	HMV	SDV	VMV[c]	: Dilution	CNV	CBV	CMV	HMV	SDV	VMV[c]	: Days : at RT[d]	CNV	CBV	CMV	HMV	SDV	VMV[c]
RT[d]	5/5	5/5	5/5	5/5	5/5	13/15	: 0	5/5	2/2	5/5	5/5	5/5	13/15	: 0	5/5	2/2	5/5	5/5	5/5	7/11
55						7/15	: 10^{-2}	4/5	2/2	5/5	5/5	4/5		: 1	5/5	2/2	4/5	5/5	5/5	7/15
57						1/15	: 10^{-2}	1/5	2/2	3/5	5/5	4/5	4/15	: 2	5/5	2/2	5/5	5/5	5/5	2/15
60	4/5		4/5	4/5		0/15	: $10^{-3.5}$						0/15	: 4	5/5	2/2	5/5	5/5	4/5	1/15
52	0/5		3/5	5/5			: 10^{-4}	1/5	2/2	1/5	1/5	1/5	1/15	: 6						0/15
54	1/5	5/5	0/5	5/5	3/5		: 10^{-5}	1/5	1/2	1/5	1/5	0/5	0/15	: 8	2/5	2/2	1/5	2/5	4/5	
66	0/5	3/5	1/5	1/5	3/5		:							: 16	2/5	2/2	1/5	0/5	0/5	
58	1/5	3/5	0/5	0/5	0/5		:							: 32	0/5	1/2	0/5	0/5	0/5	
70	0/5	3/5	0/5	0/5	2/5		:							: 71		0/2				
72	0/5	3/5			0/5		:													
74		4/5					:													
76		5/5					:													
78		5/5					:													
80		3/5					:													

[a] Test plants were *Spathoglottis plicata* except as indicated. Numerator is number of plants infected and denominator the number of plants used.

[b] Abbreviations used for virus isolates: CNV = Cattleya necrosis virus; CBV = cattleya color-break virus; CMV = Cymbidium mosaic virus; HMV = Honolono or Dendrobium mosaic virus; SDV = Spathoglottis diamond-spot virus; and VMV = Vanda mosaic virus.

[c] Previously published results (5) included for comparison. Test plant was *Vanda* sp.

[d] RT refers to room temperature, which was 20°–30°C.

PHYSICAL PROPERTIES.—The methods used to determine tolerance to heat, dilution, and aging *in vitro* were similar to those previously described (5). Unless otherwise indicated, 5 Spathoglottis plants were inoculated by the leaf-rubbing method for each treatment.

With the exception of CBV, which was not inactivated at 80° C nor after aging for 32 days, the physical properties of the isolates (Table 1) approximate those reported for Cymbidium mosaic virus by Jensen (1). The thermal inactivation point for this virus was reported to be between 65° and 70° C and its tolerance to aging longer than 7 days. Similar inactivation temperatures were obtained by White and Goodchild (13) for a Cymbidium mosaic virus in Australia.

CROSS-PROTECTION STUDIES.—In preliminary studies, groups of 5 Spathoglottis plants with systemic ringspot symptoms due to previous inoculation with CNV, CMV, HMV, or SDV were challenge inoculated with the same 4 isolates. No local lesions developed in any of the plants except in the control plants, which were healthy when inoculated with each isolate.

In a later test, CBV and VMV were included with the other isolates. Groups of 5 Spathoglottis plants were inoculated with each isolate. After the plants became systemically infected, groups were challenge-inoculated with each isolate. Top necrosis, preceded by a coarse leaf mosaic, appeared within 4–8 weeks in all series challenge-inoculated with CBV except in the series previously infected with this isolate. In the control series, CBV induced local lesions within 2 weeks and top necrosis in 3–5 weeks after inoculation. None of the other challenge isolates caused symptoms except in the control series that developed local lesions. The test was repeated, with 20 plants used for each series and challenge-inoculated with CBV. The results given in Table 2 were similar to those of the previous test.

DISCUSSION.—The similarity of symptoms caused by the isolates in most of the experimentally inoculated orchid hosts and in *D. stramonium* suggests that each isolate contains the same virus or strains of this virus. White and Goodchild (13) reported that the local-lesion reaction of *D. stramonium* 10 days after inoculation is a reliable means for the identification of Cymbidium mosaic virus. This was confirmed by Jensen and Gold (4) for a Cymbidium mosaic virus in California. Since all of the Hawaiian isolates caused similar symptoms in this host, it is probable that Cymbidium mosaic virus or its strains are involved. On some orchid genera, however, symptoms induced by an isolate were singularly different from those induced by CMV. For instance, it was noted that CBV produced a distinguishing top necrosis of Spathoglottis and a flower color break of Cattleya. Similarly, HMV caused a distinctive local lesion reaction on Cymbidium and a blossom color break of Dendrobium, whereas VMV induced a color break in Vanda. The 3 isolates caused mosaic in Cymbidium, but attempts to recover the isolates in their originating host genus resulted, in some cases, in only partial reproduction of symptoms, i.e. leaf necrosis but no flower color break. When reisolations with HMV and VMV were attempted, no symptoms were produced on Dendrobium and Vanda, respectively. This strongly suggested the presence of a second virus, in addition to Cymbidium mosaic virus, in the isolates. In a like manner, when Spathoglottis was used as the intermediate host, recovery of HMV and VMV was not obtained, although systemic symptoms were clearly evident in this host. Only CBV could be transferred from its originating host to Spathoglottis and back to Cattleya. Apparently, Spathoglottis can become infected by Cymbidium mosaic virus and by the second virus present in CBV but not by any of the other orchid viruses that might be present as a mixture in HMV and VMV. In view of these facts, the cross-protection test merely demonstrates that CBV contains a second virus not related to the Cymbidium mosaic virus apparently present in all isolates. On similar grounds, the physical properties of HMV would only represent that of a strain of Cymbidium mosaic virus. Further studies were made to determine the identity and interrelationships of the viruses involved and are reported in an accompanying article.

From the standpoint of orchid culture, the widespread occurrence of a virus that is stable and readily transmissible to several genera of orchids further emphasizes the necessity for prompt attention in the diagnosis and removal of infected plants from the nursery. In this connection, a rapid, serological method of detecting virus in orchids as described by Zaitlin et al (14) has proved useful in Hawaii (11).

TABLE 2.—*Reaction of Spathoglottis plants infected by various orchid viruses to challenge inoculation with Cattleya color-break virus (CBV)*

Virus with which plants were systemically infected[a]	No. of plants challenge-inoculated	No. of plants with top-necrosis reaction
CBV	20	0
CNV	20	10
CMV	20	11
HMV	20	8
SDV	20	11
VMV	20	9
None	20	19

[a] Abbreviations used for virus isolates: CNV = Cattleya necrosis virus; CBV = Cattleya color-break virus; CMV = Cymbidium mosaic virus; HMV = Honohono or Dendrobium mosaic vrus; SDV = Spathoglottis diamond-spot virus; and VMV = Vanda mosaic virus.

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SEROLOGICAL AND MORPHOLOGICAL RELATIONSHIPS AMONG ORCHID VIRUSES [1]

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SUMMARY

Studies confirmed the identity and prevalence of Cymbidium mosaic virus in several genera of orchids including *Cattleya, Cymbidium, Dendrobium, Spathoglottis,* and *Vanda.* A second virus previously reported only from California and found occurring as a mixture with Cymbidium mosaic virus in a bigeneric Cattleya hybrid displaying leaf mosaic and color break of the flowers was identified as Odontoglossum ringspot virus. A third virus heretofore undescribed was found associated with a strain of Cymbidium mosaic virus in Vanda Miss Joaquim affected with leaf mosaic and breaking in the color of the flowers. The rods of the new virus are shorter and somewhat more slender than those reported for other orchid viruses. Typically, they are 80 mμ in length and 13 mμ in width. This virus was found to be serologically unrelated to either Cymbidium mosaic virus or Odontoglossum ringspot virus. Four other isolates are comprised of serologically related viruses that are also similar in morphology to Cymbidium mosaic virus and are regarded as strains of this virus.

A previous study (6) demonstrated the presence, in each of 6 isolates, of a virus similar in symptomatology, host range, and physical properties to the Cymbidium mosaic virus described by Jensen and Gold (5) and by White and Goodchild (9). Three of the isolates were believed to contain only Cymbidium mosaic virus, but the studies indicated that the other 3 isolates may be composed of a mixture of Cymbidium mosaic virus and another virus. In an attempt to ascertain the identity and relationships of the viruses involved, further studies were made with precipitin tests and with the cross-absorption technique in conjunction with electron microscopy.

MATERIALS AND METHODS.—*Preparation of antisera.*—Antigens used for preparing antisera were obtained from the same sources as those previously described (6). These were the following: Cattleya necrosis virus (CNV) from a Cattleya plant with leaf necrosis; Cattleya color-break virus (CBV) from a Cattleya-Brassavola hybrid with leaf mosaic and color break of the flowers; Cymbidium mosaic virus (CMV) from Cymbidium with leaf mosaic; Dendrobium mosaic virus (HMV) from *Dendrobium superbum* Reichb. f. exhibiting both leaf mosaic and color break of the blossoms; Spathoglottis diamond-spot virus (SDV) from Spathoglottis with systemic ringspot; Vanda mosaic virus (VMV) from Vanda Miss Joaquim with leaf

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mosaic and flower color break; and healthy orchid antigen from healthy orchids of the genera represented. Frozen 5-g leaf samples were thawed and ground in a mortar with 2–3 ml of saline solution. The juice extracted through cheesecloth was centrifuged at low speed (5000 rpm) for 30 minutes after which the sample was frozen overnight. The following day, after the sample was thawed and again centrifuged at low speed, 5 ml of the clear supernatant was injected into the marginal ear vein of a rabbit. Previously, a 10-ml sample of blood to be used for normal serum was removed by heart puncture. Ten days after 5 successive weekly injections of antigen were given, blood was removed by heart puncture into a large test tube and allowed to clot. The serum was then poured off into serum bottles, labeled, and stored in the freezer to be used as needed.

TABLE 1.—*Summary of cross-absorption tests*[a] *with various isolates of orchid viruses*

Antiserum absorbed[b]	Antigen used for absorption[b]	Absorption precipitate[c]	Reaction of absorbed sera to indicated antigen[b,c]					
			CNV	CBV	CMV	HMV	SDV	VMV
cnv	CNV	++++	0	0	0	0	0	0
	CBV	++++	0	0	0	0	0	0
	CMV	++++	0	0	0	0	0	0
	HMV	+++±	0	0	0	0	0	0
	SDV	++++	0	0	0	0	0	0
	VMV	+++±	0	0	0	0	0	0
cbv	CNV	++++	0	++	0	0	0	0
	CBV	++++	0	0	0	0	0	0
	CMV	++++	0	++	0	0	0	0
	HMV	+++	0	++	0	0	0	0
	SDV	++++	0	++	0	0	0	0
	VMV	+++	0	++	0	0	0	0
cmv	CNV	++++	0	0	0	0	0	0
	CBV	+++	0	0	0	0	0	0
	CMV	++++	0	0	0	0	0	0
	HMV	+++	0	0	0	0	0	0
	SDV	+++±	0	0	0	0	0	0
	VMV	++++	0	0	0	0	0	0
hmv	CNV	++++	0	0	0	0	0	0
	CBV	++++	0	0	0	0	0	0
	CMV	+++±	0	0	0	0	0	0
	HMV	++++	0	0	0	0	0	0
	SDV	++++	0	0	0	0	0	0
	VMV	++++	0	0	0	0	0	0
sdv	CNV	+++±	0	0	0	0	0	0
	CBV	+++	0	0	0	0	0	0
	CMV	+++±	0	0	0	0	0	0
	HMV	+++	0	0	0	0	0	0
	SDV	++++	0	0	0	0	0	0
	VMV	++++	0	0	0	0	0	0
vmv	CNV	++++	0	0	0	0	0	++
	CBV	+++	0	0	0	0	0	++
	CMV	+++±	0	0	0	0	0	++
	HMV	+++	0	0	0	0	0	++
	SDV	+++±	0	0	0	0	0	++
	VMV	+++	0	0	0	0	0	0

[a] Average of 3 trials.

[b] CNV = Cattleya necrosis virus; CBV = Cattleya colorbreak virus; CMV = Cymbidium mosaic virus; HMV = Honohono or Dendrobium mosaic virus; SDV = Spathoglottis diamond-spot virus; and VMV = Vanda mosaic virus. Lower case letters, cnv, cbv, cmv, hmv, sdv, and vmv, indicate the homologous antisera for the viruses.

[c] 0 = no precipitation; + = small but definite precipitation; ++ = moderate precipitation; +++ = heavy precipitation; ++++ = very heavy precipitation; ± = slightly more than.

Serological technique.—Antigens to be tested were prepared in the same manner as for injection except that they were diluted in a ratio of 1 g of tissue to 2 ml of saline. Antisera were diluted 1 to 20 with saline prior to use. All tests involving the precipitin reaction were made by adding 0.5 ml of antiserum to an equal volume of antigen in micro test tubes supported in a specially constructed polystyrene slab in which rows of holes had been bored. The tubes were thus placed in a water bath held at 38° C for 2 hours, after which they were removed and examined. A positive test was indicated by the formation, usually within 15 minutes, of a white flocculent precipitate. If no precipitate occurred, the test was judged negative. Cross-absorption tests were made by reacting larger volumes of antigen and antiserum and then testing the supernate with additional antigen until no further precipitate occurred. The final supernate, the cross-absorbed antiserum, was then ready for use.

SEROLOGICAL TESTS.—*Dilution end-points of antigens and antisera.*—The highest dilution at which a precipitate was visible for antigens when tested with 1:20 homologous antisera was as follows: CNV (1–512), CBV (1–2,048), CMV (1–512), HMV (1–512), SDV (1–512), and VMV (1–128). With antisera (indicated in lower case), the homologous antigens were used at a 1–2 dilution. Results were as follows: cnv (1–512), cbv (1–4096), cmv (1–1024), hmv (1–2048), sdv (1–512), and vmv (1–128).

Cross-absorption experiments.—Tests established that antigens CNV, CBV, CMV, HMV, SDV, and VMV react strongly not only with their homologous antisera but also in all heterologous combinations, clearly indicating that all isolates contain a common antigenic fraction. Cross-absorption tests were then made to ascertain whether any of the isolates possess more than 1 antigenic group. Results summarized in Table 1 show that CBV and VMV contain an antigenic fraction not present in any of the other isolates. Furthermore, these 2 antigenic groups are not related to each other. This demonstrates that CBV and VMV are mixtures of 2 viruses, one of which is related to the common antigen and the other of which is not. When antisera cnv, cmv, hmv, and sdv, which had been absorbed to completion with each of the heterologous antigens, were further reacted with each of the antigens, no precipitation was observed. This indicates that these 4 isolates are antigenically related; according to Bawden (1), these would be considered as strains of 1 virus.

A previous study (6) based on the reaction of *Datura stramonium* L. indicated the probable presence of Cymbidium mosaic virus in all isolates. To establish serological evidence as to the relationship between the suspected Cymbidium mosaic virus in Hawaii and the Cymbidium mosaic virus in California, a culture of this virus was obtained from Dr. D. D. Jensen. When

a juice preparation of the culture was added to antisera of each of the Hawaiian isolates, a heavy precipitate resulted in each case. In the control, no reaction was visible in tubes containing antisera from healthy orchids. This demonstrates that the Hawaiian isolates and the California culture of Cymbidium mosaic virus have a common antigenic fraction.

ELECTRON MICROSCOPY.—Preparation of isolates for electron microscopy and measurement of virus was similar to the method described by Gold and Jensen (2). As shown in Table 2 and in Figure 1,A, the range in average size of the long rods (452–480 mμ) corresponds very closely to that reported for Cymbidium mosaic virus (2, 5). The morphology thus provides additional evidence that the virus found in all of the Hawaiian isolates is Cymbidium mosaic virus. In addition to this virus, 2 of the isolates, CBV and VMV, were each found to contain a different virus (indicated as CBV/cmv and VMV/cmv, respectively, in Table 2). This was clearly demonstrated by the use of the cross-absorption technique in conjunction with electron microscopy. Following this method, juice from CBV-infected Cattleya was reacted with an excess of cmv antiserum to agglutinate completely the Cymbidium mosaic virus component. Examination of the supernatant revealed rods averaging 291 mμ in length and straight to slightly curved in shape. The particles closely resembled the size and shape of Odontoglossum ringspot virus (4) and the rods described as occurring in a mixture with Cymbidium mosaic virus in mottled Cattleya leaves (3, 7). Two plants of *Odontoglossum grande* Lindl. were inoculated with a purified preparation of CBV containing both viruses. Within 1 week, ringspot symptoms similar to those described for Odontoglossum ringspot virus (4) were produced. Examination of the juice preparation from ringspotted leaves revealed only the short, straight or slightly curved rods but not the long sinuous rods representing Cymbidium mosaic virus. A culture of Odontoglossum ringspot virus (ORV) was obtained from Jensen and Gold and was used to prepare orv antiserum. In precipitin tests, this antiserum reacted strongly with juice extracts from either CBV-inoculated Odontoglossum or CBV-infected Cattleya. Examination in the electron microscope disclosed that the precipitate of this reaction consisted of only the short rods whereas in the supernatant, the long flexuous rods remained unaffected by orv antiserum. Conversely, when CBV from Cattleya was reacted with cmv antiserum, the precipitate contained the long flexuous rods and the supernatant, the short rods (Fig. 1.B). This establishes the

TABLE 2.—*Measurements of Hawaiian orchid viruses*

Isolate[a]	Host	No. of particles measured	Average length in mμ	Average width in mμ
CNV	Cattleya	116	452	18
CBV[b]	Cymbidium	352	463	18
CMV	Cymbidium	134	475	18
HMV	Cattleya	181	480	18
SDV	Cattleya	170	473	18
VMV[b]	Cymbidium	197	475	18
CBV/cmv[c]	Cattleya	235	291	27
VMV/cmv[c]	Vanda	381	117	13

[a] CNV = Cattleya necrosis virus; CBV = Cattleya colorbreak virus; CMV = Cymbidium mosaic virus; HMV = Honohono or Dendrobium mosaic virus; SDV = Spathoglottis diamond-spot virus; and VMV = Vanda mosaic virus. cmv = antiserum for Cymbidium mosaic virus.

[b] CBV and VMV in Cymbidium contained only Cymbidium mosaic virus.

[c] Isolates cross-absorbed with excess cmv (antiserum specific for CMV) to remove Cymbidium mosaic virus.

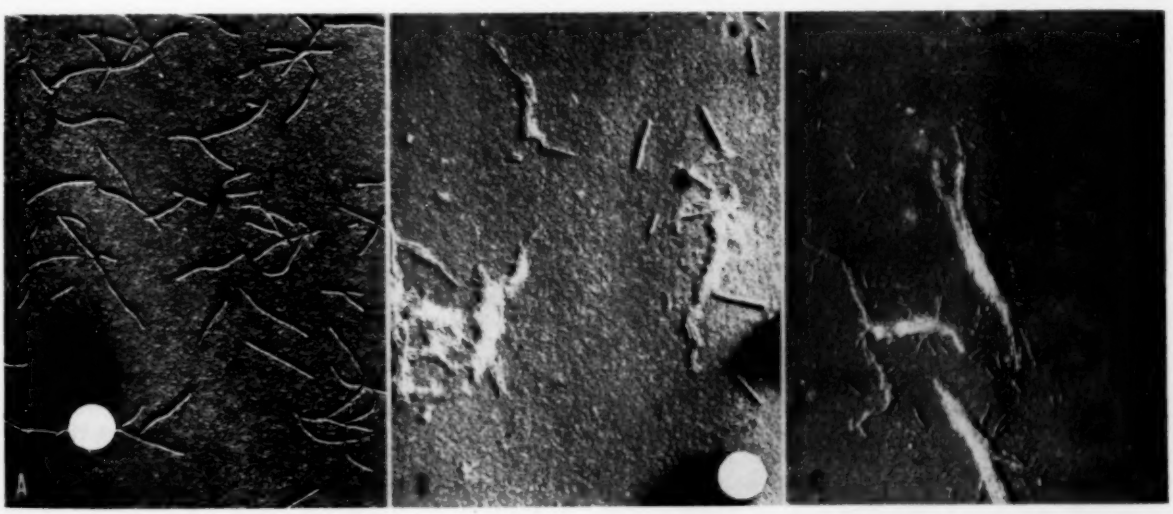

FIG. 1. Electron micrographs of orchid viruses. A) Cymbidium mosaic virus as represented in isolate CNV. B) Isolate CBV cross-absorbed with heterologous antiserum (cmv), showing agglutinated Cymbidium mosaic virus and individual particles of Odontoglossum ringspot virus. C) Isolate VMV cross-absorbed with heterologous antiserum (cmv), showing agglutinated Cymbidium mosaic virus and individual particles of a second virus component. The diameters of the polystyrene balls in A and B are 333 mμ. Magnification in C is the same as in B.

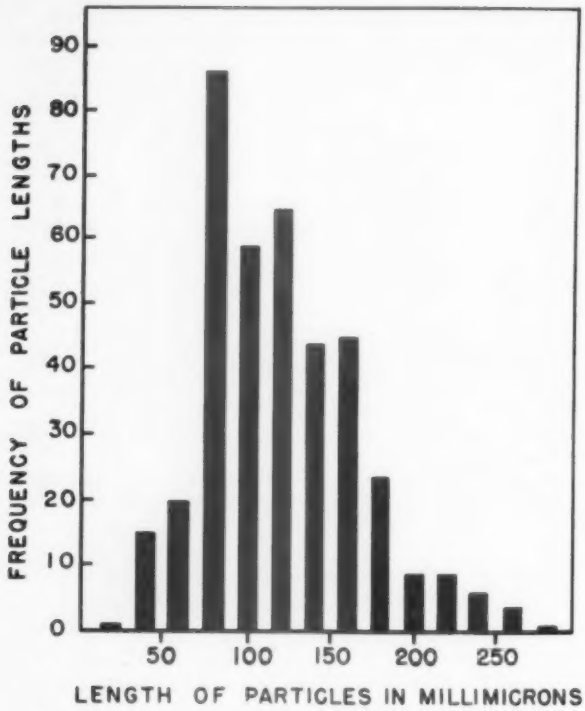

FIG. 2. Length distribution of the second virus component of Vanda mosaic virus, isolate VMV.

serological relationship between the short rods in CBV and ORV and identifies the component of CBV as Odontoglossum ringspot virus.

Similar methods were followed in determining the morphology of the second virus present in isolate VMV. Examination of VMV-cmv cross-absorption reactions revealed the presence of only Cymbidium mosaic virus in the precipitate. In the supernatant, however, the rods were different from either Cymbidium mosaic virus or Odontoglossum ringspot virus, being much shorter, straight to curved or bent, and somewhat more slender (Fig. 1,C; Table 2). The particles ranged from 20 to 280 $m\mu$ in length, with a mean length of 117 $m\mu$. Eighty per cent of the rods fell in the 80–160 $m\mu$ class, with the highest frequency occurring at 80 $m\mu$. They were uniformly about 13 $m\mu$ in width. Further reaction did not occur when the supernatant was mixed with additional cmv or with any antisera other than vmv, thus demonstrating that this virus component of VMV is serologically unrelated to any of the other orchid viruses tested. Attempts to find a host selective for this virus component alone were unsuccessful, although the Cymbidium mosaic virus could be separated from the second virus by inoculating either Cymbidium or Spathoglottis with VMV. This evidence probably explains why transmission of VMV from these hosts to Vanda could not be accomplished in a previous study (6). Apparently the presence of both viruses is required in order to cause symptoms in Vanda.

DISCUSSION.—Results of the previous study (6) indicated the likelihood that HMV also was a mixture of Cymbidium mosaic virus and a second virus, but the present studies showed that this isolate is serologically and morphologically indistinguishable from Cymbidium mosaic virus. If a mixture is assumed, 1 possible explanation for this apparently inconsistent behavior might be that the second virus is labile or present in such a low concentration that antibodies are not produced or, if produced, are formed in insufficient quantity to give a visible precipitin reaction. Such being the case, the presence of a mixture might not be detected in electron microscope preparations.

Generally, the studies demonstrate the wide orchid host range and the prevalence of Cymbidium mosaic virus in contrast to the host specificity of Odontoglossum ringspot virus and the newly described virus component in mosaic Vanda. It is interesting to note that mixtures of viruses, either demonstrated or suspected, were all associated with 3 separate flower color-break diseases of orchids. In comparison, the Cymbidium mosaic virus in Hawaii apparently can cause a variety of leaf symptoms in different orchid hosts, but it cannot by itself cause any color break of the flowers.

After this work was completed, tobacco mosaic virus was reported in Cattleya orchids in Puerto Rico (8). Based on a previous host range test (6) and on the present study, however, no evidence was found that tobacco mosaic virus is involved in any of the 6 Hawaiian isolates.

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LEAF STREAK OF ORCHARDGRASS, TIMOTHY, AND TALL OATGRASS INCITED BY SCOLECOTRICHUM GRAMINIS [1]

Samuel W. Braverman [2]

SUMMARY

Scolecotrichum graminis Fckl. is the incitant of a foliar disease of numerous grasses throughout the United States, South America, and Europe. Characteristic symptoms of the disorder are the formation of brown elongated streaks, with tufts of conidiophores developing in parallel rows in the necrotic tissue.

The causal organism overwinters on diseased tissue as old conidiophore clusters or as tiny, brown-black, subepidermal stroma that produce conidiophores and conidia in the early spring. Measurements of conidiophores and conidia and spore morphology of isolates from tall oatgrass and timothy are similar to those of isolates of the fungus from orchardgrass. Optimum temperature for mycelial growth of isolates of *S. graminis* from orchardgrass, timothy, and tall oatgrass was near 25°C. Conidial germination also was best at 25°C. Cultures of the fungus from orchardgrass, timothy, and tall oatgrass differed slightly in color and texture of mycelium. Linear growth of isolates from timothy and tall oatgrass was greater than that of isolates from orchardgrass. A method of inducing sporulation of the fungus in pure culture by ultraviolet radiation was developed; it consists of irradiating an isolate from orchardgrass, grown on a V-8 juice agar medium, for 16 minutes in covered petri dishes and 8 minutes in uncovered dishes. Isolates from each of the 3 hosts require thiamine for growth.

Several experiments involving cross inoculations to orchardgrass and timothy indicate that *S. graminis* from orchardgrass is pathogenic only to that host whereas *S. graminis* from timothy is pathogenic to both orchardgrass and timothy.

Scolecotrichum graminis Fckl. has been reported to attack over 150 grasses of both subfamilies and most of the tribes of the *Gramineae*. Included in the host range are many forage species, such as timothy (*Phleum pratense* L.), tall oatgrass (*Arrhenatherum elatius* L.), and orchardgrass (*Dactylis glomerata* L.). Morphological variants of the fungus occur (2), but little is known about their origin or whether or not they differ in pathogenicity.

Morphological, physiological, and pathological studies of several isolates of *S. graminis* from each of 3 important suscepts were made to determine whether significant differences existed among them. A method of inducing sporulation in culture and the results of life cycle studies are reported.

THE DISEASE.—The disease occurs on leaves of all ages throughout the growing season. On orchardgrass, the initial lesions appear as tiny (2 × 0.5 mm), elliptical, purplish-brown water-soaked spots with dark brown borders that are visible on both leaf surfaces. Mature lesions have been described by several workers as brown, elongated streaks (6, 7). Lesions on tall oatgrass and timothy (15–20 mm) are similar to those on orchardgrass (10–15 mm) but generally are longer.

Dense parallel tufts of conidiophores develop in older lesions in a moist atmosphere. At the close of the growing season, small, subepidermal, black, stromatic bodies, in parallel arrangement, are found in mature leaf lesions. Young lesions of streak bear a close resemblance to those incited by *Stagonospora maculata* (Grove) Sprague; however, the symptoms of the 2 diseases can be readily distinguished soon after they appear. Lesions of the former elongate, but those of the latter do not.

An additional distinguishing characteristic is the occurrence of conspicuous tufts of conidiophores or stromata in lesions of *S. graminis*, whereas lesions of *Stagonospora maculata* have no conspicuous development of pycnidia.

THE CAUSAL ORGANISM.—*Life cycle.*—Johnson and Hungerford (3) report that *Scolecotrichum graminis* overwinters readily in tufts of orchardgrass and timothy, resuming activity in the early spring. In the present study, observations were made throughout the year on orchardgrass in the field. Stromatic bodies composed of a prosenchymatous tissue were found during late autumn, winter, and early spring on leaves of orchardgrass. During late March, in Pennsylvania, the stroma that rupture the epidermis give rise to conidiophores and conidia, which serve as a source of primary inoculum on new growth. Conidia are formed throughout the growing season; upon the advent of cold weather, however, their production ceases, with the subsequent formation of the stromatic bodies. The fungus overwinters in this condition and as mycelium in diseased leaves. Sprague (8) found similar stromatic bodies on *Calamagrostis canadensis* var. *scabra* (Presl.) Hitchc. and suggests the possible ascigerous

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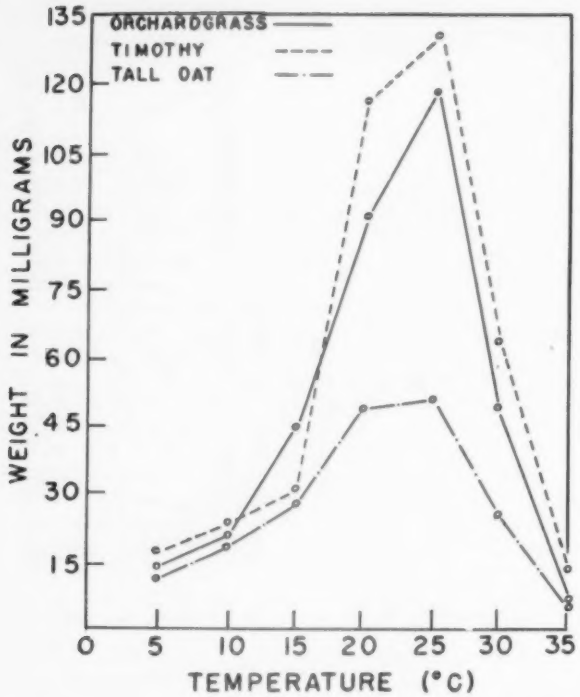

FIG. 1. Dry weight in mg of mycelium of *Scolecotrichum graminis* from 3 grass species and grown for 20 days on potato-dextrose agar. Each value represents the mean of 3 replicates.

relationship of these structures.

Morphology.—The somewhat rounded, black, stromatic bodies of *S. graminis* are approximately 0.1 mm in diameter. With the conidiophores removed, the stroma possess a much widened ostiolar-like opening that leads into a hollow, rounded cavity.

Conidiophores and conidia isolated from tall oatgrass and from timothy are similar to those described for orchardgrass by Sprague (7) and by Sampson and Western (6). The conidiophores of *S. graminis* from timothy and tall oatgrass are olive-brown, fasciculate, usually straight, and unbranched; they measure 76–105 × 7–10μ (91–8μ). The hyaline, bottle-shaped, 1- to 4-celled conidia resemble those harvested from orchardgrass. Conidia were mounted in lactophenol and 50 chosen at random from 2 collections were measured. The sizes were as follows: on orchardgrass, 29–51 × 6–11μ (42–8μ); on timothy, 31–58 × 6–10μ (42–8μ); and on tall oatgrass, 31–53 × 8–11μ (39–9μ). The measurements of conidia from these 3 hosts do not show any significant differences that might suggest a possible varietal distinction on the particular host; however, Krause (4) has described *S. graminis* Fckl. f. *avenae* Eriks., a form distinguished by its smaller conidia, on oats.

Cultural characteristics.—Mycelial characteristics of 3 monoconidial isolates of *S. graminis* from timothy, 5 from orchardgrass, and 2 from tall oatgrass varied in color, texture, and amount of growth when cultures were grown on Difco potato-dextrose agar for 1 month at 25°C. The timothy isolates were white, raised in the center, and felty, with the mycelium appressed, somewhat bayed, and cottony; diameters of growth were 39, 42, and 44 mm, respectively. The isolates from orchardgrass were white to olive-gray and somewhat raised in the center. The advanced zone was appressed, even to slightly bayed; diameters of growth were 33, 37, 31, 47, and 30 mm, respectively. The isolates from tall oatgrass were olive-gray, slightly raised in the center, and felty, with the advanced zone white, appressed, and felty to somewhat downy; diameters of growth were 43 and 47 mm, respectively.

The effect of temperature upon growth in culture.—Cultural characteristics of isolates obtained from orchardgrass and grown on Difco potato-dextrose agar varied with temperature. Below 15°C, the colonies produced a white, aerial mycelium, whereas at 15°C or above the colonies darkened and became raised in the center. The isolates from timothy and tall oatgrass were not considered in this experiment.

The effect of temperature on colony growth of isolates from tall oatgrass, timothy, and orchardgrass was determined over a range of 0° to 35°C (Fig. 1). For all isolates, the minimum temperature for growth was below 5°, the optimum 25°–28°, and the maximum above 35°.

Diseased leaves of the 3 hosts, collected in the field, were examined for the presence of conidiophores and were placed at temperatures ranging from 5° to 30°C for 72 hours. No sporulation occurred at 5°–10°, but spores were observed at 15° and were most abundant at 20°, with progressively less sporulation as the temperature was increased to 28°. No sporulation occurred above this temperature.

Spores were placed in drops of water on glass slides kept at 5° intervals from 0° to 35°C. The drops were placed inside a circle made with a glass-marking pencil to prevent spreading of the drop. Fifty spores, 10 from each of 5 fields chosen at random, were examined at each temperature. Optimum temperature for spore germination was near 25° (Table 1). In 72 hours, the conidia had germinated at 5° and after 12 hours at 35°.

TABLE 1.—*Germination of conidia of Scolecotrichum graminis at various temperatures*[a]

Temperature °C	Percentage germination after indicated no. of hours									
	2	4	6	8	10	12	24	36	48	72
0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	6
10	0	0	14	40	70	78	82	88		
15	0	12	52	76	86					
20	0	44	72	84	88					
25	0	62	88							
28	0	48	58	80	86					
30	0	6	16	24	48	62	72	82		
35	0	0	0	0	0	6	16	22	36	40

[a] Each percentage of germination is based upon 50 conidia.

Generally, in 1-septate spores, the apical cell produced a germ tube terminally, whereas the basal cell produced one laterally. Spores with 2 or 3 septa germinated apically from the terminal cell and laterally from the other cells.

Sporulation in culture.—*S. graminis* did not sporulate on ordinary laboratory media or on grain mixtures (2 parts wheat to 1 part oats). Varying the temperature and light also failed to induce sporulation. An isolate from orchardgrass, grown on a V-8 juice agar medium (1), was irradiated for 16 minutes in covered petri dishes and 8 minutes in uncovered dishes. A Cooper-Hewitt lamp (900 watts, 250 volts, 4 amperes) was used at a distance of 36 cm. Conidiophores were produced in scattered areas in the advancing mycelial zone after 10 days. Conidia that were morphologically similar to those produced in nature appeared at the apex of the conidiophores within 12 days.

Response to B vitamins.—Three isolates, 1 each from tall oatgrass, timothy, and orchardgrass, produced little or no mycelium unless vitamins were added to a glucose-asparagine medium described by Lilly and Barnett (5). Growth in the presence of thiamine was significantly greater than in the absence of vitamins. Biotin and inositol added singly and in combination to the basal medium resulted in a slight increase of growth over a medium with no vitamins added. The amount of growth obtained when all 3 vitamins were present in the medium approximated that obtained when only thiamine and biotin were present.

Inoculations.—Inoculation studies were undertaken to determine whether or not forms of *S. graminis* exist. No infection occurred when fragmented mycelial cultures were used as inoculum. Inoculum was prepared by chopping about 50 diseased orchardgrass or timothy leaves with sporulation lesions in 200 ml of tap water in a Waring Blendor for 30 seconds. The mixture was applied with an electric paint sprayer to orchardgrass, timothy, and tall oatgrass plants. The plants were then kept in a moist chamber at 100 per cent relative humidity for 48 hours at 25°C. The only infection obtained was on orchardgrass inoculated with spores from that host. When diseased excised leaves of orchardgrass (upon which spores had formed) were rubbed on living leaves of tall oatgrass, timothy, and orchardgrass, only the orchardgrass became slightly infected.

In another test, potted seedlings of orchardgrass and timothy were placed around a diseased orchardgrass plant in an inoculation chamber. Excised diseased leaves also were scattered over the seedlings. A similar procedure was carried out with a diseased timothy plant and similar seedlings. In repeated tests, spores from orchardgrass did not infect timothy, although orchardgrass was severely attacked. Spores from timothy leaves attacked that host as well as causing a mild infection of orchardgrass seedlings.

Discussion.—The several experiments have provided data that indicate that isolates of *S. graminis* from timothy, tall oatgrass, or orchardgrass were variable in culture. For example, the color of the mycelium of the timothy isolates was white as compared to the pale white-olive-gray mycelium of all orchardgrass and tall oatgrass. At temperatures optimum for growth of all isolates, the total dry weight of the mycelium of the tall oatgrass isolates was less than ½ the total dry weight of the mycelium of isolates from timothy and orchardgrass. This may be an indication of a morphological variant of the fungus on tall oatgrass.

Since the fungus would not attack timothy inoculated with spores from diseased orchardgrass under the same conditions that allowed severe infection of orchardgrass, there exists the possibility that a form distinction occurs between *S. graminis* on timothy and that on orchardgrass.

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HOST NUTRITION IN RELATION TO PHYTOPHTHORA ROOT ROT OF AVOCADO SEEDLINGS [1]

F. T. Bingham, G. A. Zentmyer, and J. P. Martin [2]

SUMMARY

Avocado seedlings were grown in nutrient solution cultures under conditions of uniform nutrition except for nitrogen, phosphorus, and potassium, which were supplied in graded amounts. *Phytophthora cinnamomi* Rands was introduced into the nutrient cultures, and the resulting development of root rot was observed. A soil experiment was performed wherein observations were made of disease development of avocado seedlings in relation to various ratios of exchangeable Ca, Mg, K, Na, and H.

Phytophthora root rot occurred in all treatments, but the progress of infection was retarded where high concentrations of N or K had been used with the solution cultures. The retarding effect may have been due to a general salt concentration rather than to association specifically with N or K. Variations in the percentages of exchangeable cations in soil had no effect on the development of Phytophthora root rot of avocado seedlings.

Adaptation of *P. cinnamomi* to such variations in environmental conditions precludes host nutrition as a practical means for control of the disease. Actually, these data show that the organism can sur ive under many soil conditions, thus signifying the threat that the disease poses to the avocado industry.

Avocado root rot, caused by the fungus *Phytophthora cinnamomi* Rands, is a major problem confronting the avocado industry in California. Undoubtedly the disease will become an important factor in avocado culture, regardless of geographical location, provided control methods are not developed. At present the disease is found in several different avocado-producing areas of the world (18, 19, 20), and, unfortunately, the causal organism is capable of attacking a variety of host plants (15).

The research work carried out at this station on the Phytophthora root rot problem has been directed toward the quest for resistant varieties of avocados (18, 19, 20, 23, 24) and for soil treatments and management practices that would control the disease. With regard to influence of environment on disease development, Zentmyer and co-workers (2, 7, 21, 22) have reported on factors such as moisture, oxygen tension, nitrite concentration, and acidity. Recently, series of greenhouse experiments were undertaken with solution cultures and soil cultures to evaluate the effect of variations in host nutrition on control of Phytophthora root rot of avocados. Results of these experiments are reported herewith.

MATERIALS AND METHODS.—Solution culture experiments were carried out in glazed ceramic cylinders holding 100 liters of aerated nutrient solution. Details of the apparatus have been described by Bingham and Zentmyer (2). One series of solution culture experiments was essentially a study of disease development under nutrient conditions of graded amounts of N, P, and K, with all the remaining plant nutrients present in amounts adequate for normal plant growth. Prior to the "seeding" of the nutrient solutions with *P. cinnamomi* they were ke,t within the range of pH 3.5 to pH 4.0. Subsequently, they were maintained at pH 4.5 ± 0.1. The following concentrations were used as variables: N—1, 70, 500, and 1000 ppm nitrate N; P—0.5, 95, 695, and 1189 ppm phosphate P; and K—1, 39, 370, 780, and 1560 ppm K. Examples are given in Table 1.

In further studies, a second solution culture technique was used wherein the avocado seedlings were preconditioned by growing them for 7 weeks in solutions from which N, P, and K had been omitted, thus producing N-, P-, and K-starved plants to be used for disease development experiments. Two days prior to "seeding," all solution cultures were renewed with a complete nutrient medium consisting of 2.5, 1.0, and 1.0 millimoles per liter, respectively, of $Ca(NO_3)_2$, $MgSO_4$, and KH_2PO_4. As in the previous experiments, the pH of the final solutions was maintained at pH 4.5 $\pm$ 0.1. Disease development was estimated by daily examination of the roots of the seedlings and by the degree of wilt exhibited by the leaves.

The soil culture experiment was an evalution of the influence of different percentages of exchangeable cations in soil on the development of Phytophthora root rot of avocado seedlings. The soil was prepared according to the procedure used by Martin and Bingham (12). Briefly, a large quantity of a recent granitic alluvial soil (Hanford series; cation exchange capacity: 9 meq/100g) was leached in 3-gal. crocks with 0.1 N HCl to make the soil predominantly H-saturated. Excess chlorides were removed by leaching with distilled water; the soil was then dried and analyzed. Bicarbonate salts of Na and K, and carbonate salts of Ca and Mg, were added to the soil in amounts calculated to produce the desired Na, K, Ca, and Mg percentages (Table 2). The salts plus a standard amount

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TABLE 1.—*Composition of nutrient solutions used in the greenhouse in the evaluation of host nutrition in relation to Phytophthora root rot of avocado seedlings*

Solution		Millimoles per liter of nutrient solution							
Material	Amount (ppm)	$Ca(NO_3)_2$	$Ca(H_2PO_4)_2$	$CaSO_4$	KNO_3	KH_2PO_4	K_2SO_4	$Mg(NO_3)_2$	$MgSO_4$
Nitrate	0	0.0	2.5	0.5	0.0	1.0	0.0	0.0	2.5
	1000	15.0	0.0	0.0	20.0	2.0	0.0	10.8	1.0
Phosphate	0	6.3	0.0	0.0	6.0	0.0	0.0	0.0	2.0
	1189	6.3	2.6	0.0	0.0	7.0	0.0	0.0	2.0
K	0	2.5	0.5	0.0	0.0	0.0	0.0	0.0	1.0
	1560	2.5	0.0	0.0	20.0	10.0	5.0	0.0	1.0
Minus N		0.0	1.0	1.0	0.0	0.0	1.0	0.0	1.0
Minus P		2.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0
Minus K		1.5	1.0	0.0	0.0	0.0	0.0	0.0	1.0
Complete		2.5	0.0	0.0	0.0	1.0	0.0	0.0	1.0

of $NH_4H_2PO_4$ were mixed into the soils; the soil was then saturated with water and incubated for 2 months in the greenhouse. After incubation, the soil was dried, mixed, and again analyzed. The ratios thus prepared are indicated in Table 2. Eight 2.7-kilo portions of each treated soil were used for the experiment. A single avocado seedling was transplanted to each pot; after several months' growth, half of each series of soils was infested with *P. cinnamomi*. The subsequent disease development was estimated by growth measurements of the shoot and of the degree of wilt.

Mexican avocado seedlings (Mexicola variety) were propagated for the solution culture and soil culture experiments by germinating seed in steam-sterilized sand. The seedlings were transplanted to the solution cultures or soil cultures when they were several inches tall. Three seedlings were placed in each of the glazed cylinders, and the replication was 3 cylinders per treatment. The soil experiment was replicated 4 times.

Inoculum for the solution cultures was prepared by growing the fungus on packets of sterilized alfalfa stems in test tubes. After 2 weeks' growth of the fungus, packets of 4–5 stems were wrapped in gauze. These gauze bags were suspended in the solution culture in the path of the circulating nutrient solution. After 4 or 5 days, lesions became visible on the roots of the seedlings. For soil infestation, a uniform quantity of a steam-sterilized mixture of wheat and oats infested with *P. cinnamomi* was placed in the soil approximately 1 in. deep and to the side of each seedling, several inches from the stem. Soil was packed over each inoculum-charged hole. As judged by wilt and growth retardation, the seedlings apparently became diseased within 4 or 5 weeks after inoculation.

RESULTS.—In the first solution culture study, all required nutrients were adequately supplied except for N, P, or K, which were supplied in amounts ranging from approximately 1 ppm to 1000–1500 ppm. The composition of the solutions was so maintained during the entire experimental period, both before and after inoculation. Five separate experiments were performed in this manner. No differential effect on disease development was noted in treatments where N, P, or K were present in concentrations low enough to be inadequate for normal plant growth (Table 3). For example, the lowest N level was maintained at a concentration of approximately 1 ppm nitrate N, low enough to cause some N-starvation symptoms to be apparent in the seedlings, yet disease developed in these cultures at rates comparable to those in treatments containing adequate nitrogen. Similarly, the low P or K treatments were without any relative effect on disease development; however, the development of Phytophthora root rot was retarded under treatments with high N or K. No differential effects were observed with the P experiment even though the amount of this nutrient ranged from 0.5 to approximately 1200 ppm phosphate P.

The second series of solution culture experiments

TABLE 2.—*Composition of soil prepared for evaluation of different ratios of cations in soil on development of Phytophthora root rot of avocado seedlings*

Series	Percentage exchangeable cation					pH[b]
	Na	K	Ca	Mg	H	
Na	1	10	65	15	9	6.3
	5	10	65	15	5	6.8
	10	10	65	15	0	7.0
	15	10	60	15	0	7.0
	20	10	55	15	0	7.2
K	1	2	65	15	17	5.8
	1	10	65	15	9	6.4
	1	20	60	15	4	6.7
	1	30	54	15	0	6.8
Ca	1	10	9	15	65	4.6
	1	10	15	15	59	4.6
	1	10	20	15	54	4.8
	1	10	30	15	44	5.1
	1	10	50	15	24	5.8
	1	10	74	15	0	6.5
	1	10	74+[a]	15	0	7.4
Mg	1	10	60	6	23	5.8
	1	10	60	10	19	5.9
	1	10	59	30	0	6.5
	1	10	29	60	0	6.2
	1	10	9	80	0	5.9

[a] An excess of Ca added as $CaCO_3$ (0.5 per cent of soil mass).

[b] Ratio of soil to water was 1:2.5.

TABLE 3.—*Development of Phytophthora root rot in avocado seedlings in complete nutrient solutions that contained increasing quantities of N, P, and K, all other nutrients being supplied in adequate amounts*

Experiment	Treatment: Material	Treatment: Amount (ppm)	Calculated osmotic pressure (atmospheres)	Percentage root rot after indicated no. of days[a]: 4	6	8	10	12	14
1	Nitrate N	1		6	52	82	95	98	98
		70	0.3	7	44	54	78	87	94
		500	1.6	0	1	3	30	59	84
		1000	3.0	0	1	1	8	27	64
2	Nitrate N	70		14	59	89	99	100	100
		140		39	89	98	100	100	100
		210		18	77	93	99	100	100
		500		8	60	87	96	98	100
3	Phosphate P	0.5		18	48	82	87	93	98
		95	0.3	13	43	75	81	94	93
		695	1.0	1	15	40	63	83	92
		1189	1.1	2	30	69	82	91	94
4	K	1		30	67	90	97	99	100
		39	0.3	55	86	97	99	100	100
		390	0.7	27	75	95	98	99	100
		780	1.1	3	29	81	87	95	99
		1560	2.1	7	44	86	97	99	100
5	K	39		40	75	89	91	95	99
		1560		1	4	7	29	47	60

[a] Root rot determined by visual examination of roots and estimation of percentage of roots affected.

involved the preconditioning of avocado seedlings to low N, P, or K nutrition before inoculation. This was done by growing seedlings for 7 weeks in nutrient solutions that lacked N, P, or K. Two days prior to seeding, all solutions were renewed with a single complete solution. As a control, plants were cultured continuously in solutions identical with those used during the period when the solutions were being seeded with *P. cinnamomi*. As in the previous experiments, no differential effects on the rate of disease development were observed (Table 4). At the time of inoculation, the plants grown in minus-N solution were extremely yellow, showing symptoms of acute nitrogen starvation. The plants grown in minus-P or minus-K solutions were smaller than the control plants (complete nutrient medium) and presumably were under stress because of deficiencies of P or K. No real difference occurred subsequently in the rate at which the plants became diseased.

None of the soil treatments were especially effective in retarding disease development (Table 5). Possibly the highest addition of Na delayed slightly the rate at which the plants manifested symptoms of Phytophthora root rot.

DISCUSSION.—These studies indicate that control of Phytophthora root rot through regulation of host nutrition or alteration of the composition of soil by changing the content of Na, K, Ca, or Mg would not be feasible. Actually, they demonstrate the adaptation of the pathogen to a wide variety of possible soil-chemical conditions. The disease is thus exceedingly difficult to control. Other investigators have observed that diseases of certain plants could be retarded or accelerated by regulation of host nutrition. For example, Castano and Kernkamp (6) found that *Rhizoctonia solani* Kühn readily attacked soybeans under conditions of deficiencies of Ca, Mg, Fe, S, N, or P. Walker and co-workers (8, 14, 16, 17) have shown with a variety of vegetable plants that the activity of several diseases could be altered by regulating the composition of the nutrient solution.

Nutrition experiments with little-leaf disease of shortleaf pine are especially interesting in view of the fact that the causal agent of avocado root rot is the primary pathogen responsible for the pine disease (3, 4, 5). Hepting and co-workers (9, 10, 13) were able to reduce the activity of the little-leaf disease with N fertilization. They also noted that diseased trees con-

TABLE 4.—*Development of Phytophthora root rot in avocado seedlings that were grown for 7 weeks in —N, —P, —K, or complete nutrient solutions prior to inoculation, after which they were grown in complete nutrient solution*

Treatment prior to seeding	Solution seeded with *P. cinnamomi*[a]	Percentage of root rot after indicated no. of days[b]: 5	8	11	14	17
Minus N for 7 weeks	—	0	0	0	0	0
	+	3	35	66	87	97
Minus P for 7 weeks	—	0	0	0	0	0
	+	1	19	54	90	97
Minus K for 7 weeks	—	0	0	0	0	0
	+	11	41	59	92	99
Complete nutrient solution	—	0	0	0	0	0
	+	6	20	58	94	98

[a] A gauze bag containing inoculum of *P. cinnamomi* grown on sterilized alfalfa stems was suspended in the nutrient solution.

[b] Root rot determined by visual examination of roots and estimation of percentage of roots affected.

TABLE 5.—*Growth of avocado seedlings in soil in relation to infestation of soil with Phytophthora cinnamomi and to different percentages of exchangeable cations in the soil*

Percentage of exchangeable cation in soil	Growth of inoculated series,[a] expressed as a percentage of growth of noninoculated series, when percentage of exchangeable cations was as indicated			
	Na	K	Ca	Mg
1	58			
2		27		
5	41			
6				12
9			29	
10	51	33		17
15	46			
20	80	26	21	
30		26		17
50			32	
60				27
74			34	
80				18
150			30	

[a] Average growth of 4 plants relative to that of 4 noninoculated plants, after 7 weeks.

tained lower amounts of Ca than were found in healthy trees.

Anderson (1), investigating root rot of pineapple plants grown in nutrient solutions, noted that high salinity and high K reduced the degree of infection by *P. cinnamomi*. These results coincide with those from the solution culture experiments described by the present authors. In the solution culture experiments, however, salinity may be operative in reducing activity of the fungus. Calculations of the osmotic pressures (Table 3) reveal that the highest values were obtained for those treatments that were associated with the lowest rates of disease development.

The soil experiment with exchangeable cations demonstrated no benefit from K, even though K was increased from 2 to 30 per cent on the exchange complex. Soils prepared in this manner would be relatively salt free. This would support the contention that high salinity, and not K *per se*, retarded disease development on plants in the solution culture. The form of nitrogen apparently is unimportant too, because in a previous study, Bingham and Zentmyer (2) observed avocado root rot occurring at equal rates when either NO_3^- or NH_4^+ was used.

Hydrogen-ion concentration, at least under nutrient solution conditions in the greenhouse (2), controls the rate of development of Phytophthora root rot of avocados. Such extremes in pH are required, however, that successful field acidification for control is unlikely. Perhaps with other plants such as the pineapple plant (11) soil acidification has a place. The present authors have a number of field plots in diseased avocado groves where various soil amendments (sulfur, lime, etc.) are being tested, but as yet no definite conclusions can be drawn.

Zentmyer (18, 20), in preliminary experiments, has noted some benefit through the use of alfalfa meal as a soil amendment. In view of the nutritional studies reported herein, it would appear that any benefit derived from alfalfa meal is not due to changes in the nutrition of the host. Perhaps the microfloral composition was altered in such a manner that the pathogenicity of *P. cinnamomi* was reduced.

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SNOW MOLD OF FORAGE CROPS IN ALASKA AND YUKON[1]

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SUMMARY

In May, 1956, a survey was made of winter injury to forage crops in Alaska and Yukon. Twenty-eight samples of diseased plants were taken at various locations throughout these territories. Isolations from these samples were made at the Plant Pathology Laboratories at Edmonton and Lethbridge, Alberta. The low-temperature pathogens isolated were *Sclerotinia borealis* Bub. & Vleug., *Plenodomus meliloti* Dear. & Sanford, *Typhula idahoensis* (Fr.) Ces., and an unidentified basidiomycete, the principal snow-mold pathogen in western Canada. This basidiomycete had not previously been found elsewhere. Many additional organisms were recovered from the samples, including several *Fusarium* spp., but the above-mentioned were the only ones identified as low-temperature pathogens. Much of the winter-killing of herbaceous plants in Alaska and Yukon was caused by these fungi, but it was noted that *Medicago falcata* L. survived the winter conditions better than did any other forage crop examined.

The severe winters in Alaska and Yukon prevent the production of most of the common perennial grasses and legumes that are grown throughout western Canada. It has been known for some time that snow mold is responsible for a great part of the losses in forage crop production in western Canada and in the Baltic area, but information had not been obtained on this disease in Alaska and Yukon. Dr. E. L. Nielson of the U. S. Department of Agriculture, after visiting Alaska in 1955, suggested that surveys should be conducted to ascertain whether low-temperature pathogenic organisms were present, and, if so, what damage to forage crops was caused by them.[4] The present authors conducted such a survey in 1956, and an additional survey in Yukon was made by the senior author the same year.

Materials and methods.—Eight days were spent examining winter damage to forage crops in Alaska and Yukon in May, 1956. A distance of approximately 1200 miles was covered in this survey, and 28 samples of diseased plants were taken at various locations. Isolations of the low-temperature organisms were made from these samples at the Plant Pathology Laboratories at Edmonton and Lethbridge.

Diseased portions from the roots, crown, and shoots were plated on potato-dextrose agar and incubated at 2°C. Surface sterilization of some of the infected material was necessary in order to isolate the low-temperature pathogens, and the method described by Schein and Kereto,[5] by use of ethyl alcohol and sodium hypochlorite, was adopted. It was particularly effective in obtaining pure cultures of *Sclerotinia borealis* Bub. & Vleug.

Results and discussion.—The low-temperature pathogens recovered from forage crops at the various locations are listed in Table 1. Many additional organisms were isolated from these samples, including several *Fusarium* spp.; however, those recorded are the only ones that were identified as low-temperature pathogens.

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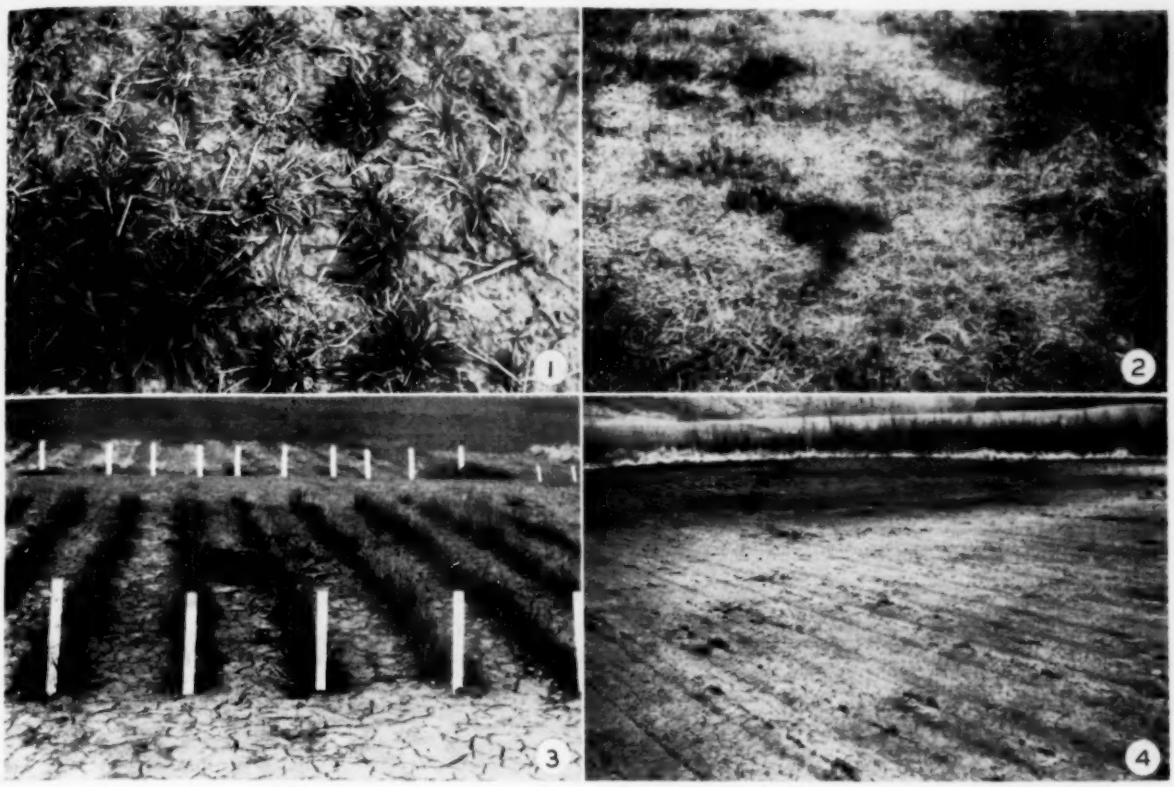

FIG. 1-4. FIG. 1. Damage on *Secale cereale* (winter rye) caused by *Sclerotinia borealis*. College, Alaska.—FIG. 2. Severe snow mold damage to a mixture of *Bromus inermis* and *Poa pratensis*, caused by *Sclerotinia borealis*. College, Alaska.—FIG. 3. Dead rows of *Melilotus alba* killed by a combination of low temperatures and *Plenodomus meliloti*. Experimental Farm, Whitehorse, Yukon.—FIG. 4. A field of *Phleum pratense*, showing little survival after being exposed to low temperatures at Palmer, Alaska, where the winds have removed the protective snow cover during the winter.

Plenodomus meliloti Dearn. & Sanford, a root-rot pathogen of sweetclover and related crops,[6] was isolated from *Festuca rubra* L. and *Poa pratensis* L. in Alaska (Table 1). It appeared to be the predominant fungus in the tissues of the roots from these 2 samples. It is likely, however, that the fungus was saprophytic or weakly parasitic on tissues that had been damaged by low temperatures or by other pathogens.

The low-temperature basidiomycete previously reported from Alberta[7] was found for the first time beyond the Canadian border. One of the isolates of the low-temperature basidiomycete was recovered from native grass in the mountains of the Alaska Range and the other at College, Alaska, from cultivated grass. Although no fruiting structures have ever been discovered on this species, it is unlikely that such wide distribution could be accomplished without a spore form. Isolates of the low-temperature basidiomycete from Alaska have the same cultural appearance as those obtained in western Canada.

It was apparent that a large amount of the winter-killing of herbaceous plants in northern Alaska and

TABLE 1.—*Low-temperature pathogens isolated from forage crops in Alaska and Yukon*

Low-temperature pathogen	Location	Host
Low-temperature basidiomycete	College, Alaska	*Poa pratensis*
	Summit Lake, Alaska	*Calamagrostis canadensis* (Michx.) Beauv.
Sclerotinia borealis	College, Alaska	*Bromus inermis*
	College, Alaska	*Phleum pratense* L.
	College, Alaska	*Secale cereale*
Plenodomus meliloti	Fairbanks, Alaska	*Festuca rubra*
	College, Alaska	*Trifolium hybridum*
	Palmer, Alaska	*Poa pratensis*
	Whitehorse, Yukon	*Melilotus alba*
	Mile 970, Yukon (Alaska Highway)	*Trifolium pratense*
Typhula idahoensis	Whitehorse, Yukon	*Poa pratensis*

[6] Sanford, G. B. 1933. A root rot of sweet clover and related crops caused by Plenodomus meliloti Dearness and Sanford. Can. J. Research 8: 337-348.

[7] Broadfoot, W. C., and M. W. Cormack. 1941. A low-temperature basidiomycete causing early spring killing of grasses and legumes in Alberta. Phytopathology 30: 1058-1059.

Yukon was caused by low-temperature pathogens. *S. borealis* was prevalent in the Tanana Valley and caused severe damage to *Secale cereale* L. (winter rye), *Bromus inermis* Leyss., and *Poa pratensis* L. (Fig. 1, 2). *P. meliloti* caused extensive damage in Yukon to *Melilotus alba* Desv. (Fig. 3), *Trifolium pratense* L., *T. hybridum* L., and *Medicago sativa* L. *P. meliloti* was also found parasitizing the wild legume *Hedysarum mackenzii* Richards. No evidence of the disease was found on *Medicago falcata* L.

Typhula idahoensis Remsberg was recovered from large, typical snow-mold patches on *P. pratensis* at Whitehorse. No evidence of this fungus was found in the extreme northern districts where *S. borealis* was apparent. This was not too surprising, since Jamalainen[8] reported *S. borealis* as the principal snow-mold pathogen in the northern parts of Finland and Sweden. *Typhula* spp. and *Fusarium nivale* (Fr.) Ces. occur farther south in the Scandinavian countries.

S. borealis, *T. idahoensis*, and the unidentified low-temperature basidiomycete cause rotting of the crown and bud tissues, and the plants are killed out in large patches of irregular shape. The symptoms of attack by the 3 pathogens were so similar that it was necessary to isolate the causal fungus before making the differentiation. *P. meliloti*, in contrast, infected the roots of the plants rather than the crowns and buds, and the infection was general throughout the fields.

Some snow-mold damage was observed in the Matanuska Valley, but the great loss to forage crops in this area was caused by desiccating winds and exposure of the plants to low temperatures (Fig. 4). The protective snow layer is removed by winds that sweep through the valley at extremely high velocities. *M. falcata* is the only cultivated legume that consistently survives these severe winter conditions.

The resistance of *M. falcata* to low temperatures, desiccation, and low-temperature pathogens is of basic interest and suggests need for investigation into the nature of this resistance.

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A METHOD FOR STUDYING ABSORPTION OF STREPTOMYCIN BY USING LEAF DISKS OF SEDUM PURPUREUM[1]

John L. Lockwood[2]

SUMMARY

Detached leaves of *Sedum purpureum* Tausch. were sprayed on the underside with streptomycin preparations, then placed on water agar in petri dishes. After 24 hours, disks were cut from the leaves, the lower epidermis was stripped off, and the disks were frozen. Estimations of streptomycin content were made by assaying the disks by an agar-diffusion method with a sensitivity of 0.1 μg. Assays of juice from leaves sprayed with streptomycin gave values about twice those of assays of leaf disks, necessitating transformation of leaf disk values. A 2-fold difference in antibiotic concentration from a true mean was discovered by use of 6 replications of 1 leaf disk each. Approximately 14 per cent of the streptomycin deposited was absorbed from aqueous sprays, whereas about 49 per cent was absorbed from sprays containing Tween 20, a surface-active agent. Similar proportions of streptomycin were absorbed from small or large deposits. More streptomycin was absorbed by leaves incubated in a moist atmosphere than by leaves allowed to dry rapidly. Leaves incubated in darkness absorbed as much streptomycin as did leaves incubated in light. Leaf age and portion of the leaf sampled were important sources of variability in leaf-disk assays. Disks from washed leaves with epidermis intact and disks from leaves stripped of epidermis gave similar assay values.

Interest in systemic fungicides and bactericides in plant pathology has necessitated the development of methods for studying absorption of these materials by plant leaves. The most commonly used method involves washing the treated parts and assaying the expressed juice (3, 5). This method requires 2 assumptions: 1) that all the material on the leaf surface is

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washed off and 2) that none of the material that has penetrated into the leaf is leached out by the washing process. Furthermore, variation within the relatively large samples required cannot be determined. The method described herein was devised as an alternative method and avoids these limitations. Leaf disks from *Sedum purpureum* Tausch., a plant with an easily removed lower epidermis, are used.

MATERIALS AND METHODS.—*Assay methods.*—Assays of streptomycin were made by an agar-diffusion method. The assay medium was composed of the following ingredients: Difco dehydrated nutrient broth, 8 g/l; boric acid, 2 g/l; trisodium phosphate, 4 g/l; agar, 20 g/l. The ingredients, with the exception of trisodium phosphate, were mixed and autoclaved. Two ml of a 50 per cent (W/V) solution of trisodium phosphate was added to the melted agar at about 50° C to give a pH of 8.2. Use of this pH level resulted in inhibition zones of maximum diameter; higher pH levels inhibited growth of the test bacterium.

The test bacterium, *Agrobacterium tumefaciens* (E. F. Sm. & Town.) Conn was maintained on yeast-extract agar (per 1: yeast extract, 2 g; glucose, 10 g; agar, 20 g) at 28°. Bacterial cells from a 24-hour culture were mixed with sterile distilled water, and the mixture was diluted such that it gave a colorimeter reading of 85 per cent light transmission at 525 mμ. One part of the suspension was diluted in 50 parts of melted and adjusted agar at 42°. Three hundred ml of seeded agar, containing 3.4×10^7 cells, was poured into each of the required number of large pans similar to those described by Lockwood et al (8).

Crystalline streptomycin sulfate was used in all experiments. Assay standards were prepared by diluting a stock solution prepared in 0.1 M phosphate buffer at pH 6.5 in a 2-fold series with buffer. One-tenth ml of these solutions was pipetted onto filter paper disks (Schleicher & Schuell No. 740-E) and dried. Slopes and positions of dosage-response curves made with dry disks did not differ from those made with wet disks. Streptomycin solutions used for spraying plants were prepared in water or in 0.1 per cent Tween 20 (polyoxyethylene sorbitan monolaurate) solution. Concentrations of 200 and 1000 μg of streptomycin per ml usually were employed.

Assays of streptomycin in leaf tissue.—Greenhouse-grown clonally propagated *S. purpureum*, a succulent herb with an easily removable lower epidermis, was used as the test plant. Leaves were detached, and the basal ends were sealed with petrolatum. The lower sides of leaves were sprayed with streptomycin solutions by means of an atomizer attached to an air line. The leaves were placed sprayed side up on water agar in closed petri dishes. After 24 hours, disks were removed from the leaves with a cork borer (½ in. diameter), which was rinsed and dried after each boring. The lower (sprayed) epidermis was removed from each disk, and the disks were frozen until assayed. Disks were assayed by placing them on the surface of the assay agar, sprayed side down, while still frozen. In most experiments, 12 leaves were used for each treatment, and 1 disk from the same position of each leaf was assayed.

Analysis of variance was applied to the leaf disk data; zone diameters were used as the basis of calculations. The dosage-response curves as plotted on semilogarithmic paper were straight lines, and both position and slope varied but little from experiment to experiment. A difference of 4 mm in zone diameter generally represented a 2-fold difference in streptomycin concentration. The amount of streptomycin deposited on and absorbed by the leaves was expressed as μg per cm^2 of leaf tissue.

When leaf juice was to be assayed, the disks with lower epidermis removed were frozen, then ground in a mortar containing a small amount of water. The total fresh weight of the disks for each treatment was determined beforehand. The mixture was centrifuged, and 0.1 ml of centrifugate was applied to filter paper disks and assayed. Dry weights of similar disks showed that leaves contained 95 per cent moisture. Data, as μg of streptomycin per ml of leaf juice, were transformed to μg per cm^2 of leaf tissue for comparison with values obtained by assays of disks. The formula used was $X = \frac{J}{0.95} \times W$, where J = μg of streptomycin per ml of leaf juice and W = weight in grams of 1 cm^2 of leaf.

Deposition.—The amount of streptomycin deposited on leaves of *S. purpureum* was determined by assaying filter paper disks placed among the leaves to be sprayed. Paper disks and leaf disks of the same diameter sprayed with various concentrations of streptomycin gave inhibition zones of the same diameters. Careful spraying resulted in a uniform deposit.

RESULTS.—*Replication.*—The numbers of leaf disk samples necessary to discover given magnitudes of difference from a true mean were calculated. A mean zone diameter was determined from several experiments. Estimates of variance of this mean were calculated with data from 7 separate experiments in which 12-disk replications were used. The mean estimate of variance, 7.8, was used as an estimate of the true variance. Alpha (risk of rejecting a true hypothesis) and beta (risk of accepting a false hypothesis) were set at 0.05. When these values are used, the detection of differences of 1, 2, 3, 4, and 5 mm from a true mean would require, respectively, 100, 25, 11, 6, and 4 leaf disks. It was desired that the leaf-disk assay detect a 2-fold (4 mm) or smaller difference in antibiotic concentration. Since a difference of 3 mm could be detected by 11 disks, and this number was not excessive, a minimum of 11 was used. Further increase in precision would require a disproportionate increase in the number of disks.

Effect of pH on leaf-juice assay.—Assays of antibiotics in leaf juice or in leaf disks may be influenced by hydrogen ion concentration or by other factors in the juice. Comparisons were made between the dosage-response curves of a dilution series of streptomycin in the following solvents: 1) 0.05 M phosphate buffer

at pH 6.5, 2) distilled water, 3) unbuffered leaf juice (mixture of 1 part leaf juice and 1 part distilled water) at pH 4.4, 4) leaf juice prepared with 0.05 M phosphate buffer at pH 4.4, 5) leaf juice prepared with buffer at pH 7.0, and 6) leaf juice prepared with buffer at pH 9.0.

When assays of a test solution were made immediately after preparation, the dosage-response curve did not differ, either in position or in slope, from that obtained when assays were made after the test solution had stood at room temperature for 3 hours. Leaf juice or buffer without streptomycin gave no zones of inhibition. Results indicated that assays of streptomycin in leaf juice were unaffected by hydrogen ions or by other constituents of the leaf juice and that reliable assays of streptomycin in leaf juice could be made when buffered standards were used.

Absorption of streptomycin by leaves of S. purpureum.—The amount of streptomycin absorbed from sprays by detached leaves of *S. purpureum* was determined by corresponding assays of leaf disks and of leaf juice in 11 separate experiments. The mean deposit was 2.4 μg per cm^2 of leaf surface. The mean amount of streptomycin absorbed as determined by assays of leaf juice was 0.52 μg per cm^2 or 21.2 per cent of that deposited. The mean amount absorbed as determined by leaf-disk assays was 0.27 μg per cm^2 or 11.2 per cent. Inasmuch as leaf-juice values probably were more valid estimates of streptomycin content of leaves than were leaf-disk values, the relationship between leaf-juice (X) and leaf-disk (Y) values was determined so that disk values might be converted to juice values (Fig. 1). The correlation coefficient for juice and disk values was 92 per cent, indicating a close relationship between the variates. $\hat{X}$, the predicted X, was calculated by the formula $\hat{X} = 0.04 + 1.77\ X$, which is derived from $\hat{X} = \overline{X} - B\ (\overline{Y} - Y)$, where B, the slope of the line, was 1.77. Disk values were converted graphically or by the formula in subsequent experiments.

It was observed that larger amounts of streptomycin were absorbed by the leaves if the streptomycin solutions were prepared with 0.1 per cent Tween 20 than when prepared with water alone. Regression lines showing the relation between deposit and absorption (with data from 11 experiments in which 0.1 per cent Tween 20 was used as the solvent and 9 experiments in which water alone was used as the solvent) are plotted in Figure 2. Correlation coefficient for deposit and amount of streptomycin absorbed from the Tween 20 solutions was 85 per cent; that for the tests with water alone as the solvent was 87 per cent. These values indicate a close relationship between the 2 variates. A mean of 49 per cent of the streptomycin applied with Tween 20 was absorbed by Sedum leaves; a mean of 14 per cent was absorbed when water alone was used as the solvent.

There was no correlation between deposit and percentage of streptomycin absorbed. Correlation coefficient for experiments in which Tween 20 was used was —2.2 per cent; that for experiments without Tween 20 was 4.4 per cent.

The effect of a moist atmosphere on absorption of streptomycin by leaves of *S. purpureum* was tested in 2 experiments. Leaves were sprayed, then placed on water agar in open or closed petri dishes. Leaves in closed dishes retained a film of moisture for several hours, whereas those in open dishes dried quickly. Assays of leaf disks made after 24 hours showed that approximately twice as much streptomycin was absorbed by leaves in closed dishes as in open dishes (Table 1), suggesting that the retention of moisture on the leaf surface favored absorption of streptomycin by the leaves. Closed dishes were used in subsequent tests.

The influence of light and darkness on absorption of

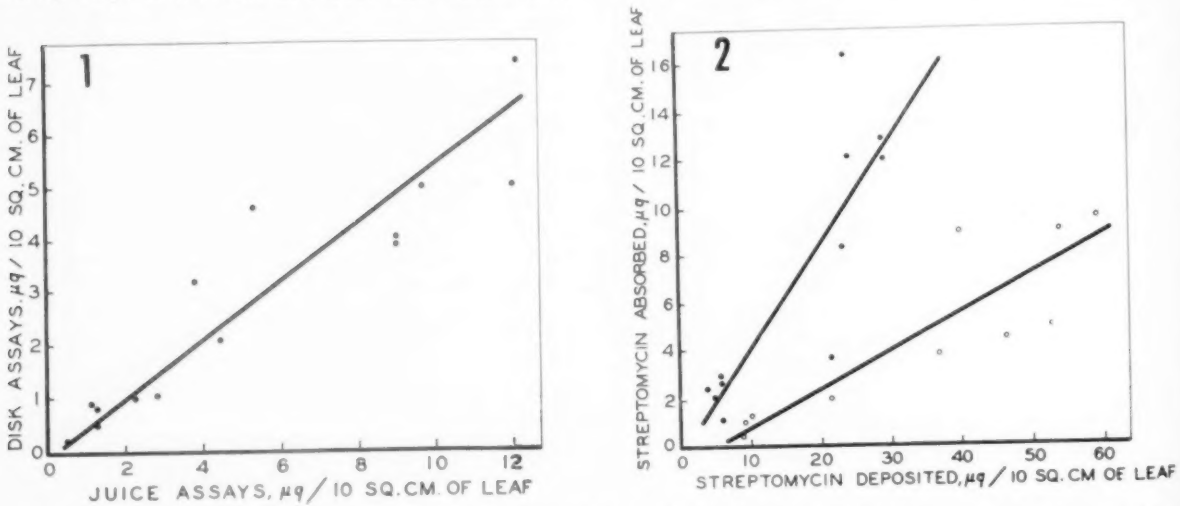

FIG. 1-2. FIG. 1. Relation between estimates of streptomycin absorbed by leaves of *Sedum purpureum* as determined by assays of leaf disks and of leaf juice.—FIG. 2. Relation between amounts of streptomycin deposited on and absorbed by leaves of *Sedum purpureum* from solutions prepared with 0.1 per cent Tween 20 with water alone; • = solutions prepared with Tween 20, ○ = solutions prepared with water alone.

TABLE 1.—*Comparative absorption of streptomycin by detached leaves of Sedum purpureum incubated 24 hours on water agar in open and in closed petri dishes*[a]

Test	Streptomycin deposited per 10 cm² of leaf surface	Streptomycin absorbed per 10 cm² of leaf surface[b]	
		Closed dishes	Open dishes
	μg	μg	μg
1	4.8	2.0	1.1**
	29.0	12.8	6.3**
2	5.7	2.7	1.5**
	23.0	8.4	3.7**

[a] Streptomycin solutions were prepared in 0.1 per cent Tween 20. Estimations of streptomycin were made by assays of leaf disks by means of an agar-diffusion method. Each value is based on assays of 1 disk from each of at least 12 leaves.

[b] Double asterisks indicate statistically significant difference at 1 per cent level between values for open and closed dishes.

streptomycin by detached leaves of *S. purpureum* was tested in 1 experiment. Leaves were sprayed with streptomycin solutions, then placed in petri dishes, half of which were placed in darkness and half under a fluorescent lamp for 24 hours. Plants had been exposed to 4 hours of daylight before leaves were detached. There was no significant difference in the amounts of streptomycin absorbed by leaves exposed to light or to darkness.

Variable plant factors.—Three variable plant factors, test plant, leaf age, and portion of the leaf sampled, were studied to determine their contribution to variation in leaf-disk assays. Leaves of a similar age from 4 different plants were sprayed to determine if the plants differed in ability to absorb streptomycin. Assays of disks from the same portion of each leaf showed no significant differences in amounts of streptomycin absorbed by leaves from the 4 plants.

The absorbency of young and old leaves of *S. purpureum* was tested. Disk samples from the same relative position of each leaf were assayed. In 2 of 3 experiments, more streptomycin was absorbed by older leaves than by young leaves, whereas in the third the reverse was true (Table 2). The absorbency of terminal and of basal portions of leaves of a similar age was tested. In 2 of 6 tests, more streptomycin was found in terminal disks, whereas in 2 other tests more was found in basal disks (Table 3). There was no statistically significant difference in amounts of streptomycin in terminal or basal disks in 2 other experiments. Because of the inconsistency of the results, no conclusions could be drawn with respect to the absorbency of leaves of different ages or of different portions of the leaves. The results suggest, however, that treatments should be applied to leaves of the same age or apportioned systematically among leaves of different ages and that similar portions of leaves should be sampled.

TABLE 2.—*Comparative absorption of streptomycin by young and old detached leaves of S. purpureum incubated 24 hours on water agar in Petri dishes*[a]

Test	Streptomycin deposited per 10 cm² of leaf surface	Streptomycin absorbed per 10 cm² of leaf surface[b]	
		Young leaves	Old leaves
	μg	μg	μg
1	5.8	1.8	2.6**
	23.0	4.8	8.8**
2	4.0	2.2	2.6*
3	21.1	9.0	6.6*

[a] Streptomycin solutions were prepared in 0.1 per cent Tween 20. Estimations of streptomycin were made by assays of leaf disks by means of an agar-diffusion method. Each value is based on assays of 1 disk from each of at least 12 leaves.

[b] Single asterisk indicates statistically significant difference at 5 per cent level between values for young and old leaves. Double asterisks indicate statistically significant difference at 1 per cent level between values for young and old leaves.

TABLE 3.—*Comparative absorption of streptomycin by basal and terminal portions of detached leaves of Sedum purpureum as determined by assays of leaf disks incubated 24 hours on water agar*[a]

Test	Streptomycin deposited per 10 cm² of leaf surface	Streptomycin absorbed per 10 cm² of leaf surface[b]	
		Terminal portion	Basal portion
	μg	μg	μg
1	10.0	1.7	2.0*
	46.0	3.4	5.3*
2	4.8	2.0	2.7**
	29.0	12.8	16.0**
3	5.8	2.5	1.9**
	23.0	8.0	5.6**
4	16.0	5.0	3.4**
5	6.0	3.2	3.6
	24.0	12.8	13.0
6	4.0	2.4	2.5
	24.0	16.0	16.0

[a] Streptomycin solutions were prepared in 0.1 per cent Tween 20 except for Test 1, in which water only was used. Each value is based on assays of 1 disk from each of at least 12 leaves.

[b] Single asterisk indicates statistically significant difference at 5 per cent level between values for basal and terminal disks. Double asterisks indicate statistically significant difference at 1 per cent level between values for basal and terminal disks.

Comparison of assays of stripped leaf disks with washed leaf disks.—The absorption of streptomycin by leaves of *S. purpureum* was determined by assaying disks with the epidermis removed and disks from washed leaves with the epidermis intact. Uniform leaves of the same age were sprayed and incubated for 24 hours. Half the leaves were prepared for assay by stripping the epidermis. The other half were washed for 20 minutes under tap water, but the epidermis was not stripped. Disks were cut from similar leaf portions for both treatments, then frozen and assayed. The differences between the amounts of streptomycin detected by the 2 methods were not statistically significant (Table 4). Thus, the washing method appeared

TABLE 4.—*Comparison of leaf-washing and leaf epidermis-stripping techniques for determination of streptomycin absorption by detached leaves of Sedum purpureum incubated 24 hours on water agar in petri dishes*[a]

Test	Streptomycin deposited per 10 cm^2 of leaf surface[b]	Streptomycin absorbed per 10 cm^2 of leaf surface[b] when indicated type of leaf was assayed	
		Washed leaves	Stripped leaves
	μg	μg	μg
1	5.7	2.4	2.7
	23.0	7.4	8.4
2	6.1	2.9	2.1
	29.0	12.5	12.8

[a] Streptomycin solutions were prepared in 0.1 per cent Tween 20. Estimations of streptomycin were made by an agar-diffusion method. Each value is based on assays of 1 disk from each of at least 12 leaves.

[b] Differences between values for washed and stripped leaves were not statistically significant.

to be satisfactory under the conditions of these tests.

DISCUSSION.—The methods described in this report should be useful for studies on the absorption of streptomycin by plant leaves. That this antibiotic is absorbed by plant tissues has been reported previously (3, 4, 5, 9). Little is known, however, of the influence of such factors as environment and chemical additives on the absorption of this or other antibiotics or of the mechanisms of absorption of streptomycin by leaf tissues. The method should also be useful in screening tests for determining the permeability of leaf tissue to candidate systemic fungicides and bactericides.

The use of leaf disks (7, 12) permits the assay of small samples and provides a ready estimate of variability, advantages not inherent in the leaf-juice method. If leaf age and disk position, important variable factors, are standardized, the leaf-disk method can give reasonably precise results without excessive replication or labor. The sensitivity of the assay method (minimum limit approximately 0.1 μg) permits the use of dosages approximating those that might be used in field applications. The use of detached leaves facilitates experimentation and allows standardization of the antibiotic deposit, so that amount absorbed can easily be related to deposit in comparisons of various treatments. Leaves of *S. purpureum* are sufficiently succulent to retain turgidity throughout the experiments.

Inconsistent results in experiments to determine the relative absorbency of young and old leaves and of terminal and basal portions of leaves are difficult to understand. Equally puzzling is the finding (1) that slightly less schradan, a systemic insecticide, is absorbed by old chrysanthemum leaves than is absorbed by young leaves and that least is absorbed by leaves of middle age. Absorbency of leaves of different ages and of different portions of the same leaf possibly is influenced to a greater extent by environmental conditions under which the plant is grown than by leaf age or leaf portion *per se*. Perhaps variation within and between experiments could be reduced with better control of soil moisture and temperature.

The data indicate that similar results can be obtained by assaying disks from washed leaves with the epidermis intact and disks from which the epidermis has been removed. Nevertheless when washed leaves are used, one must be certain that the material on the leaf surface is removed and that the absorbed antibiotic is not leached by the washing process. By assaying disks from leaves with the epidermis removed one can eliminate these possible sources of error. It is no more difficult to remove the epidermis from leaves of *S. purpureum* than to wash them.

The finding that leaves kept in a moist atmosphere absorbed more streptomycin than those allowed to dry is similar to the results of Gray (5), who found that hygroscopic agents such as glycerol increased the absorption of streptomycin by bean leaves. Similar increases in absorption of 2,4-D with hygroscopic agents have been noted (2, 11). These agents probably enhanced absorption by retaining moisture on the leaf surface, thus permitting absorption for a longer time (2, 5, 11). The surface-active agent Tween 20 increased absorption of streptomycin by leaves of *S. purpureum*, a confirmation of Gray's finding (5). Surface-active agents also enhanced absorption of 2,4-D by plant leaves (6). Electron microscope studies on the submicroscopic structure of plant surfaces emphasize the importance of the spreading of the film of moisture so that it is in close contact with the cuticle; polystyrene macromolecules applied as sprays with surface-active agents were in close contact with the cuticle whereas without surface-active agents the macromolecules were high up on the wax groups, which are superimposed on the cuticle (10).

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RELATION OF SLIME PRODUCTION TO MECHANISM OF WILTING AND PATHOGENICITY OF PSEUDOMONAS SOLANACEARUM[1]

Akhtar Husain and Arthur Kelman[2]

SUMMARY

Culture filtrates of a highly pathogenic strain of *Pseudomonas solanacearum* caused wilting of tomato cuttings, whereas culture filtrates of weakly pathogenic and avirulent strains did not. A heat-stable, viscous substance capable of causing reversible wilting of tomato cuttings was obtained by alcoholic precipitation from the culture filtrate of the virulent strain. This substance was not present in the culture filtrates of weakly pathogenic or avirulent strains. Preliminary chemical tests indicated that the substance was a complex polysaccharide of a high molecular weight with glucose as a main component. By means of an eosin-serum stain technique, it was possible to demonstrate the presence of bacterial slime around cells of the pathogenic strain obtained from cultures on solid media and in bacterial exudate from the vascular tissues of diseased plants. In the stained preparations, the distinctive reticulate pattern that characterized the slime around cells of the pathogenic strain in culture and in the host also characterized the heat-stable wilt-inducing material obtained from liquid cultures of this strain. No slime was observed around cells of the weakly pathogenic or nonpathogenic types. Tomato cuttings wilted in tracheal sap that was obtained from diseased tobacco and tomato plants and freed of bacteria by filtration; cuttings placed in tracheal sap from healthy plants did not wilt. Following inoculation with the virulent strain, Rutgers tomato plants wilted within 1 week. Although the weakly pathogenic strain did not cause wilt and multiplied only to a limited extent, infected plants did show symptoms of stunting. It was concluded that the extracellular polysaccharide or slime produced by the virulent strain is the primary wilt-inducing factor. This slime apparently increases the viscosity of the vascular stream and interferes with the movement of water in the vessels. The weakly pathogenic and avirulent strains produce no slime, and their limited multiplication in host tissues may be related to the inability to form slime.

The causal agent of southern bacterial wilt or Granville wilt, *Pseudomonas solanacearum* E. F. Sm., is one of the most destructive bacterial plant pathogens in the warm regions of the world (14). The main characteristic effect of *P. solanacearum* is the sudden wilting of infected plants. The mechanism by which this vascular parasite causes wilting in plants has been a controversial subject for a number of years. Two main theories have been offered to explain the wilting mechanism in this disease (14). According to 1 theory, wilting of plants is caused by occlusion of water-conducting tissue by masses of bacteria (7). According to the second theory, initially presented by Hutchinson (13), wilting results from the production by the pathogen of a systemic toxin that disturbs the osmotic system of the leaves. The paper by Hutchinson is frequently cited as the first report that attributed pathological wilting of plants to the formation of a toxic substance by a vascular parasite. Hutchinson based his conclusions on an experiment in which wilting resulted when an aqueous solution of an alcoholic precipitate from a bouillon culture of the bacterium was introduced into a tobacco plant.

A detailed study of the mechanism of wilting by *P. solanacearum* was carried out by van der Meer (29), who found water movement to be less rapid in diseased plants than in healthy plants. Movement of eosin dye was very irregular in diseased plants, whereas dye movement was regular and uniform in healthy

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plants and healthy parts of the diseased plants. Excised wilted leaves and terminal shoots of diseased plants recovered when placed in water. In an investigation of the toxin theory, a 2-day-old beef broth culture filtrate of the bacterium was introduced into tomato and tobacco cuttings. No wilting occurred within 12 hours, but gums were observed in transverse sections of the treated cuttings. Van der Meer concluded that the primary factors in wilting were 1) the damage to root tissue, 2) the formation of gums and tyloses in the vessels, and 3) the plugging of vessels by the bacteria. Gum formation was interpreted as a host response to the action of injurious compounds produced by the bacterium.

A second intensive investigation on the possible causes of wilting was completed by Grieve (7). Histological examinations of diseased plants revealed that wilting could not be attributed solely to formation of tyloses or gums. When excised diseased tomato shoots were placed in a solution of eosin red, no dye (or a very limited amount of dye) moved up the shoots. In addition, transpiration and water uptake were significantly slower in diseased than in healthy plants. Grieve concluded that wilting of infected tomato and potato plants resulted from gradual mechanical plugging of vessels by masses of bacteria rather than by the formation of gums, tyloses, or toxins.

Recently Hutchinson's concept of toxigenic wilting has been supported by Kunz (19). Two toxic fractions were obtained by alcoholic precipitation from 2- to 5-month-old beef broth cultures of the bacterium. It was suggested that the first substance was a bacterial slime (a polypeptide complex) that caused wilting by its physical action in plugging the water-conducting tissue of the plant. The second toxic substance was considered to be a plasma poison that caused wilting by destruction of the semipermeable property of the plasma membranes in a manner similar to that postulated for lycomarasmin by Gäumann (6). Kunz also suggested that the bacterial slime probably was converted to toxic substances by the action of the enzymes from the host plant.

Among the explanations for the divergent results obtained in the above studies on mechanism of wilting is the possibility that pure virulent clones of *P. solanacearum* were not maintained, particularly when liquid cultures were grown for long periods of time. Smith (27) and many other investigators (14) have observed that this bacterium loses pathogenicity rapidly in culture; however, the basis for this rapid attenuation was not known. The relationship of pathogenicity to colony types was first studied by Okabe (21, 22), who noted the appearance of colony type variants that differed greatly in morphology from the virulent type. Apparently, loss in pathogenicity of cultures of *P. solanacearum* on certain culture media can be associated with the development of high populations of weakly virulent or avirulent variants of the bacterium (16). Furthermore, there is now available a differential medium on which the different colony types can be distinguished readily (15).

These findings indicated the advisability of an investigation of the mechanism of wilting in which a study would be made of the role of metabolic products of pathogenic and weakly pathogenic strains of *P. solanacearum*. The primary objective of the present study was to test the validity of the toxin theory; for this purpose strains of the wilt bacterium differing in pathogenicity were compared for the formation of wilt-inducing substances in culture and for specific effects on host plants. A preliminary report on these studies has been made (11).

MATERIALS AND METHODS.—One highly pathogenic strain (designated as F-1) and 2 variant strains (designated as B-1 and B-2) of *P. solanacearum* were used. Strain F-1 was isolate No. 60 originally obtained from naturally infected tomato in 1953 (15). The B-1 (nonpathogenic) and B-2 (weakly pathogenic) variants were derived from a single-cell culture of the F-1 strain. The colonies of these 3 strains grown on a peptone-casein-hydrolyzate medium containing 2,3,5-triphenyltetrazolium chloride had the following characteristics: F-1 = fluidal, irregularly round, smooth, convex, and initially white with a pink center; B-2 = butyrous, irregularly round, slightly rough, raised, and deep red with a narrow white border; and B-1 = butyrous, round, small, smooth, slightly convex, and deep red with a narrow white border (Fig. 2, E, F, G). The mean diameters of the colonies of the 3 types after 72 hours growth on the tetrazolium medium were as follows: F-1, 4.1 mm; B-2, 2.5 mm; and B-1, 1.4 mm. Cultures were purified by streaking on the peptone-casein-hydrolyzate medium containing tetrazolium chloride and selecting well-isolated colonies. Following purification stock cultures of the 3 strains were prepared by suspending 5 loops of bacterial growth in 5 ml of sterile distilled water in capped test tubes. These suspensions were stored at 25°C. Bacterial cultures were grown in a liquid medium of the following composition: ammonium sulfate, 1.32 g; magnesium sulfate, 0.08 g; zinc sulfate, 8 mg; ferric chloride, 2.5 mg; manganese sulfate, 1.8 mg; sucrose, 12 g; yeast extract, 1.0 g; casein hydrolyzate (Difco), 2.5 g; pH 7.0 phosphate buffer, 10 ml; and distilled water, 1000 ml. Yeast extract and casein hydrolyzate were dialyzed in 500 ml of distilled water at 5°C for 24 hours, after which the other ingredients were dissolved in the resulting dialyzate and the solution made up to 1 liter with distilled water.

Bottom Special tobacco (*Nicotiana tabacum* L.) or Rutgers tomato (*Lycopersicon esculentum* Mill.) plants grown in soil or sand were used as host plants in the inoculation experiments. Cuttings of Rutgers tomato seedlings were used for laboratory experiments. The seedlings were grown in sand and watered with a mineral nutrient solution. Unless otherwise indicated, all experiments to determine wilt induction in cuttings were carried out in the following manner. Uniform seedlings were removed from sand, and the roots were thoroughly washed in tap water. To minimize contamination, the basal portions of the seedlings were dipped in 70 per cent ethanol for 2 minutes and rinsed

in sterile tap water to remove the alcohol. The stems were severed under sterile tap water and the cut ends immersed in the various test solutions. Test solutions were placed in test tubes (25 ml) or vials (10 ml and 3 ml), depending on the size of cuttings. Containers were sterilized prior to use, and sterile cotton plugs were inserted in the mouths of the tubes to minimize contamination. Cuttings in solution were held in a lighted chamber at 30°C and at a relative humidity of approximately 50 per cent. At the end of the designated test periods, the cuttings were examined for symptoms of wilting and rated on an arbitrary scale as follows: 0 = no wilting, 1 = slight drooping of lower leaves, 2 = wilting of lower leaves, 3 = wilting of all the leaves except the terminal bud, 4 = complete wilting without desiccation of leaves, and 5 = complete wilting of all leaves and terminal portion of stem with desiccation of leaves. After the wilt ratings were made, the basal one-third of each stem was trimmed off and the cuttings transferred to distilled water so that possible recovery of turgor could be noted. All experiments on cuttings were repeated at least 3 times.

In studies on the effect of culture filtrates on cuttings, the bacterial growth from a 48-hour potato-dextrose agar slant culture was suspended in 10 ml of sterile distilled water and added to 1 liter of medium in a 2-liter flask. After 24–48 hours of growth at 32°C on a rotary shaker, the bacterial cells were removed by centrifuging at 5000 rpm for 1 hour. The supernatant liquid was sterilized by passage through ultrafine sintered glass filters. One-half of the sterile culture filtrate was autoclaved at 18 lb. pressure for 20 minutes. Twenty-ml portions of the test solutions were placed in sterile 25-ml tubes and cuttings of 5- to 6-week-old tomato seedlings with 3 or 4 well-expanded leaves were placed in the test solutions. Five cuttings were placed in each solution; sterile distilled water and the sterile culture medium were used as controls. All tests were repeated 3 to 5 times.

In the preparation and isolation of the wilt-inducing principle, 4 liters of 48-hour-old liquid shake cultures of the F-1 and B-2 strains and 4 liters of the sterile medium were used. The bacterial cells were removed from the liquid cultures by centrifugation in an air driven Sharples supercentrifuge. The clarified culture medium was concentrated to 1/20 of its original volume by vacuum distillation at 55°C. The concentrated culture filtrate was centrifuged at 5000 rpm for 1 hour to remove any residual bacterial cells or other solid materials. To the viscous supernatant, 2 volumes of absolute ethanol were added and the amorphous precipitate was allowed to settle for 12 hours at 5°C. This initial precipitate, designated as Fraction I, was collected by centrifugation and dried under vacuum. The clear supernatant was again concentrated to its original volume by distilling off the alcohol under vacuum. The reconcentrated solution was poured into 4 volumes of absolute ethanol. This second precipitate, designated as Fraction II, was allowed to settle for 12 hours at 5°C, collected by centrifugation, and dried under vacuum.

Each of the dried precipitates was ground to a powder and dissolved in distilled water at concentrations ranging from 0.1 to 0.5 per cent. One portion of the 0.5 per cent solution of the wilt-inducing Fraction II obtained from the virulent (F-1) strain was autoclaved at 18 lb. pressure for 20 minutes, and a second portion was dialyzed against distilled water for 24 hours. All these solutions were tested on 6-in. tomato seedlings. Sterile distilled water and a solution of the precipitate obtained from the sterile medium served as controls.

In the investigation of the chemical nature of the wilt-inducing substance, a 2 per cent solution of the alcoholic precipitate was hydrolyzed in 1 N sulfuric acid in a boiling water bath for 12 hours. The hydrolyzate was neutralized with barium carbonate, and the precipitate formed was removed by centrifugation. Both the nonhydrolyzed and the hydrolyzed material were analyzed qualitatively for carbohydrate and protein by the methods described by Hawk et al (8). Since this preliminary qualitative analysis indicated that the substance was a polysaccharide in nature, an attempt was made to identify the sugar constituents by 1-dimensional descending paper chromatography. A 4:1:5 mixture of butanol, acetic acid, and water and a 9:1:5 mixture of butanol, ethanol, and water were used as solvents (23). The spots were made visible by spraying with aniline phthalate (24) and heating the paper in an electric oven at 105°C for 5–10 minutes.

In experiments to determine whether wilt-inducing substances were produced *in vivo*, 12- to 14-week-old tomato and tobacco plants grown in 6-in. pots were used. When the plants were 12 to 18 in. in height, they were inoculated with the F-1 strain by forcing a needle into the stem through a drop of bacterial suspension placed in a leaf axil near the top of the stem. After 10–12 days, when the plants were severely wilted, they were removed from the pots and the roots washed free of soil with tap water. The roots were then placed in glass jars containing water, the top portions of the stems were cut off, and the stumps were attached to glass tubes by means of rubber tubing. The tracheal sap of the plants was collected in the glass tubes by applying a negative pressure of approximately 0.5 atmospheres. Sap obtained from individual diseased plants was pooled and centrifuged at 20,000 rpm for 20 minutes to remove bacterial cells. Tracheal sap of healthy plants of the same age and size was obtained in a similar manner. The supernatant fluid was passed through ultrafine sintered glass filters to remove any remaining bacterial cells. The bacteria-free sap was divided into 2 portions; 1 portion was heated in an autoclave at 18 lb. pressure for 20 minutes and the other portion was not heated. Cuttings from tomato seedlings (25–35 days old and 4-in. tall) were transferred to vials containing 2 ml. of the sap. Five cuttings were used for each test solution, and sterile distilled water was used for the control.

To investigate the specific effects of different strains of *P. solanacearum* on a susceptible host, tomato plants were inoculated with the highly virulent (F-1) and

weakly pathogenic (B-2) strains. Ten 8-week-old tomato plants approximately 12 in. in height were inoculated in the lower portion of the stem with each strain of the bacterium; the needle-puncture technique described above was used. To avoid contamination, the inoculation court was surface-sterilized prior to inoculation and covered with a sterile cotton pad after inoculation. After 1 week, the plants were examined for wilting symptoms, isolations were made from selected stem segments, and similar segments were examined histologically.

For histological studies, stem segments approximately 1 cm in length were taken from the apical portion of the plants and fixed in a solution of formalin, acetic acid, and ethanol for 48 hours. The fixed material was dehydrated by passage through 1-butanol series and embedded in paraffin. Transverse sections, 10–12 μ thick, were cut with a rotary microtome and stained with safranin and fast green. The stained material was examined to determine the extent of multiplication and invasion of inoculated plants by the bacteria.

Results.—*Effect of culture filtrate on cuttings.*—Tomato cuttings placed in the culture filtrate of the highly virulent strain (F-1) exhibited wilting symptoms after 1½ hours and were completely wilted at the end of 5 hours (Fig. 1,A). Initially, the petioles of lower leaves started drooping; flagging of all the leaves and of the terminal portions of the cuttings soon followed. The first symptom on the leaf blade was an upward curling of the leaf margins; this was followed by complete loss of turgor and shriveling of the leaves. Necrosis of margins and interveinal areas also was observed. At the end of 12 hours, the petioles and the apical portions of the stems had collapsed and most of the leaves were desiccated. In addition to the above foliage symptoms, seedlings placed in the nonheated culture filtrate showed a pronounced softening of the basal portion of the stem immersed in the solution. In contrast to the effects produced by the nonheated culture filtrate, cuttings placed in the heated culture filtrate first showed symptoms of wilting after 5 hours and were completely wilted after 12 hours; however, there was little injury to leaves, and the terminal buds were not affected. Furthermore, no evidence of maceration of tissues was noticed in these cuttings.

No wilting developed in cuttings placed in culture filtrates of the nonpathogenic (B-1) and weakly pathogenic (B-2) strains (Fig. 1,A), but the bases of cuttings placed in nonheated culture filtrates of these strains were severely macerated. Those placed in heated filtrates were not. A severe epinasty of leaves and twisting of terminal portions of stems was evident in cuttings in both heated and nonheated culture filtrates of these strains.

It was noted in these initial tests that the culture filtrate of the highly pathogenic strain was much more viscous than the culture filtrates of the less pathogenic strains. Therefore, it seemed advisable to make exact measurements of the relative viscosity of these culture filtrates, and Fenske-Ostwald viscometers (size 100) were used for this purpose.

The wilt-inducing effect of the heated and nonheated culture filtrates was determined in conjunction with viscosity measurements. All cuttings placed in the test solutions were rated for wilting according to

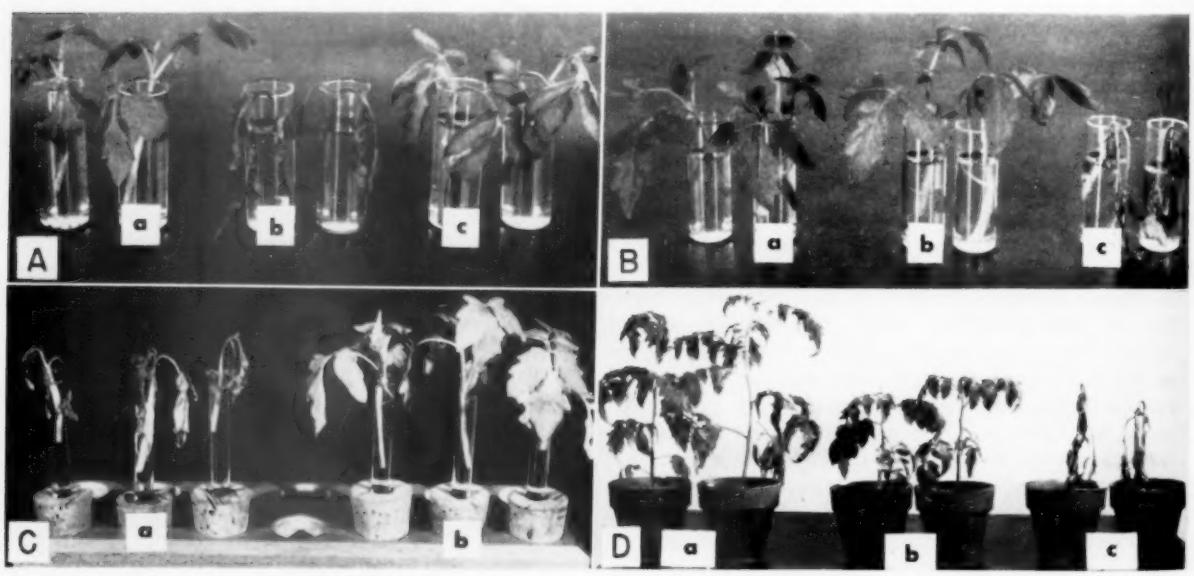

Fig. 1. A) Effects on tomato cuttings of a) sterile medium, b) culture filtrate of a highly pathogenic strain, and c) a weakly pathogenic strain of *Pseudomonas solanacearum*. B) Effects on tomato cuttings of alcoholic precipitate from a) sterile medium, b) weakly pathogenic strain, and c) highly pathogenic strain of *P. solanacearum*. C) Effects on tomato cuttings of tracheal fluid from a) diseased tomato plants infected by the F-1 strain and b) healthy tomato plants. D) Symptoms produced on tomato by different strains of *P. solanacearum:* a) noninoculated control, b) weakly pathogenic B-2 strain, and c) highly pathogenic F-1 strain.

TABLE 1.—*The relative viscosity and wilt-inducing effect of heated and nonheated culture filtrates of different strains of Pseudomonas solanacearum*

Test solution	Relative viscosity[a]	Wilting index[b]
Culture filtrate of F-1	83.3	5
Heated culture filtrate of F-1	83.0	3.5
Culture filtrate of B-1	50.5	0
Heated culture filtrate of B-1	50.4	0
Culture filtrate of B-2	51.5	0
Heated culture filtrate of B-2	50.5	0
Sterile medium	50.0	0

[a] Relative viscosity expressed in terms of flow time in seconds in size 100 Fenske-Ostwald viscometers.

[b] Average wilting index for 5 cuttings after 12 hours (0 = no wilting, 5 = complete wilting).

the arbitrary scale described previously. These data and the viscosity measurements are presented in Table 1. Results confirmed the preliminary observations of differences in viscosity, since the filtrate of F-1 was found to be significantly more viscous than were the filtrates of the other strains. Wilting of cuttings occurred only in the filtrates of highest viscosity. Wilting index was slightly lower in the heated culture filtrate of the F-1 strain than in the nonheated filtrate.

When bases of the stems of wilted cuttings were clipped off and the cuttings transferred to water, those cuttings that had been placed in nonheated culture filtrates recovered only partially. Cuttings transferred from heated culture filtrates recovered completely.

Isolation of the wilt-inducing material.—A flocculent precipitate (Fraction I) was obtained by adding 2 volumes of absolute ethanol to the culture filtrates of F-1 and B-2 as well as to the sterile medium. When 4 volumes of alcohol were added to the concentrated culture filtrate of F-1 after the removal of Fraction I, a gelatinous precipitate (Fraction II) was obtained. This precipitate remained suspended and formed a semisolid gel when it was kept at 5°C for 12 hours. In contrast, after addition of alcohol to the concentrated culture filtrate of B-2 and the sterile culture medium, a small amount of a flocculent precipitate formed, and all of it settled to the bottom within a few minutes. Fraction II obtained from the culture filtrate of F-1 gave an opalescent viscous solution in water; Fraction I from F-1 and Fractions I and II from B-2 and the sterile culture medium produced transparent and nonviscous solutions (Table 2). When Fractions I and II from culture filtrates of F-1 and B-2 and the sterile medium were tested on cuttings, only Fraction II from F-1 caused wilting (Fig. 1,B). This Fraction II of F-1 filtrate at a concentration of 0.1 per cent caused tomato cuttings to wilt within 5–6 hours; no wilting was caused by Fraction II from B-2 or the sterile culture medium even at a concentration of 0.5 per cent (Table 2, Fig. 1,B). Fraction II from F-1 was heat stable, but lost some wilt-inducing capacity when a 0.5 per cent solution was dialyzed against distilled water for 48 hours. Wilting caused by this substance was reversible, since wilted cuttings recovered completely when the bases of cuttings were clipped off and the cuttings transferred to distilled water.

The presence of a highly viscous heat-stable material in the culture filtrates of the pathogenic strain indicated the possibility that this strain formed capsular material or extracellular slime. To investigate this possibility, cells of the 3 strains were stained by the India ink method and also by the eosin-serum method of Howie and Kilpatrick as recommended by Duguid (5) for staining bacterial capsules and slime. Examination of slides of the F-1 strain prepared from a 72-hour culture on a peptone-casein-hydrolyzate medium revealed that this strain formed slime in abundance on this medium (Fig. 2,A). Slime was not present in similar preparations of either strain B-1, or B-2 (Fig. 2,B). When a solution of the partially purified wilt-inducing substance obtained from the F-1 strain was stained by the eosin-serum technique, the distinctive reticulate pattern formed by this substance on the slide (Fig. 2,C) was the same as the reticulate pattern of the slime around the cells of the F-1 strain taken from culture. This indicates that the heat-stable viscous material precipitated from the culture filtrate of the pathogenic strain is similar to or identical with the slime that is formed by the cells of this strain.

Identification of the wilt-inducing fraction.—The alcoholic precipitate (Fraction II) of the F-1 strain was subjected to a number of qualitative tests for protein and carbohydrate; the data are summarized in Table 3. These results, as well as data on other physical characteristics, indicate that this fraction can be considered to be a polysaccharide; however, the material also gives a positive test with the Biuret and Millon's reagents, indicating that nitrogenous groups are also present. Nitrogen is commonly present in most complex bacterial polysaccharides (17).

In the chromatographic analyses of the acid hydrolyzate of Fraction II of the F-1 strain, only glucose was detected. This was confirmed by qualitative tests for glucose on the hydrolyzate. No amino sugars were detected when the paper strips were sprayed with Morgan and Elson's reagent as modified by Partridge

TABLE 2.—*Wilt-inducing effect and viscosity of solutions of the precipitate (Fraction II) obtained from culture filtrates of virulent (F-1) and weakly virulent (B-2) strains of Pseudomonas solanacearum*

Source of precipitate	Relative viscosity[a]	Wilting index when concentration in per cent[b] was as indicated		
		0.5	0.2	0.1
Culture filtrate of F-1	165	5	3	2
Culture filtrate of B-2	52.5	0	0	0
Sterile medium	52.0	0	0	0

[a] Viscosity of 0.5 per cent solution expressed as flow time in seconds in size 100 Fenske-Ostwald viscometers.

[b] Mean wilting index of 5 cuttings after 12 hours (0 = no wilting, 5 = complete wilting).

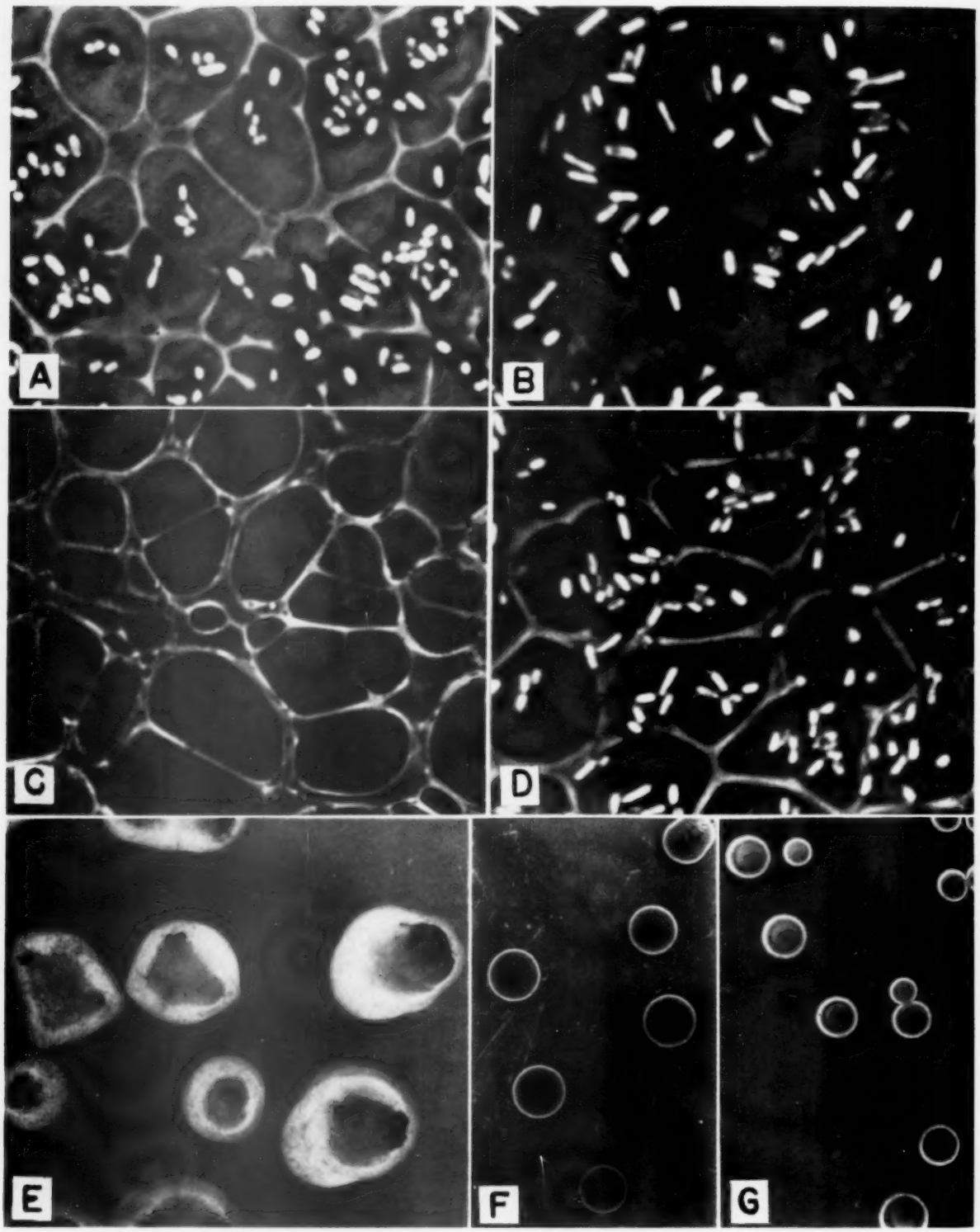

FIG. 2. A-D) Phase contrast photomicrographs (2000×). A) Cells and slime of the highly pathogenic F-1 strain of *Pseudomonas solanacearum* prepared from culture on a solid medium. B) Slime-free cells of the weakly pathogenic B-2 strain prepared from culture on a solid medium. C) Slime isolated from the culture filtrate of the highly pathogenic F-1 strain. D) Cells and slime of the highly pathogenic F-1 strain from a diseased tobacco plant. E-G) Five-day-old colonies of 3 strains of *P. solanacearum* on tetrazolium medium (2×). E) Highly pathogenic F-1. F) Weakly pathogenic B-2. G) Nonpathogenic B-1.

TABLE 3.—*Results of qualitative tests on the wilt-inducing substance obtained from culture filtrate of the highly pathogenic F-1 strain of Pseudomonas solanacearum*

Tests	Non-hydrolyzed	Hydrolyzed
Molisch reaction	+	+
Fehling's test	−	+
Benedict's test	−	+
Xanthoproteic reaction	−	−
Biuret test	+	+
Millon's reaction	+	−
Ninhydrin test	−	−

(23). The possibility exists that other constituents in addition to glucose and nitrogenous components also are present in Fraction II of the F-1 strain.

Production of wilt-inducing substances in vivo.—Considerable difficulty was experienced in obtaining an adequate volume of tracheal sap by applying suction to the basal portions of the stems of diseased tomato and tobacco plants. The yield of liquid from a diseased plant was usually less than 1/10 the volume of sap obtained from a healthy plant. This observation serves to support the concept that wilting in diseased plants occurs because passage of water is prevented by the occlusion of the vascular tissues.

Cuttings placed in tracheal sap obtained from infected tomato and tobacco plants began to wilt after 1 hour and were completely wilted after 5 hours (Fig. 1,C). Leaf margins and tips showed severe necrosis after 12 hours. In contrast, no wilting had developed in cuttings placed in tracheal sap of healthy tomato and tobacco plants after 12 hours. The toxic substance present in the tracheal sap was heat stable, since the wilt-inducing potential was not appreciably reduced by autoclaving at 18 lb. pressure for 20 minutes. Similar results were obtained in each of the 3 experiments.

To determine whether the slime formed by the bacterium in culture was also produced in infected plants, bacterial exudates obtained from cut stems of diseased tobacco and tomato plants were stained by the eosin-serum method. Slides prepared from these exudates (Fig. 2,D) showed more slime than did those from cultures on solid media (Fig. 2,A). These results are similar to those obtained with certain animal pathogens, since organized slime layers in the form of capsules are larger in the bacterial smears prepared from infected hosts than in those from cultures on solid media (4). The reticulate pattern of the slime noted in preparations from cultures on solid media (Fig. 2,A) was also apparent in the slides with bacteria from diseased plants (Fig. 2,D).

Inoculation experiments with different strains.—Tomato plants inoculated with the weakly pathogenic (B-2) and with the highly pathogenic (F-1) strain were examined for wilting symptoms after 1 and 2 weeks. Plants inoculated with the weakly pathogenic (B-2) strain showed no symptoms of permanent wilting after 2 weeks (Fig. 1,D). Initially, slight drooping of leaves was observed at midday in plants inoculated with the B-2 strain; however, the plants always recovered at night. Although wilting symptoms did not develop in these plants, stunting and leaf epinasty were evident. Adventitious roots formed on the stems, which became greatly swollen at the point of inoculation. In addition, vascular tissues were discolored above and below the inoculation court. All plants inoculated with the F-1 strain were severely wilted in 7 days; stems were completely collapsed, and plants were usually dead in 12 to 14 days.

When isolations were made from terminal portions of plants inoculated with these strains, the bacteria were obtained consistently from the apical sections. Only the weakly pathogenic (B-2) type was reisolated from plants inoculated with the B-2 type. Isolations from plants inoculated with the F-1 strain occasionally yielded the B-2 as well as the F-1 type. The number of B-2 colonies present in these isolations from plants inoculated with F-1 usually represented less than 10 per cent of the total population.

When paraffin and free-hand sections of infected stems were examined, disorganization of vascular bundles was observed in plants inoculated with each of the strains; however, the F-1 strain (Fig. 3,A) caused greater disorganization of vascular tissue than did B-2 (Fig. 3,B). In plants infected by either strain, xylem vessels filled with bacterial masses often were noted. In certain sections, cavities in the stem were also filled with bacteria; however, in plants infected with B-2, cavities were smaller in number and size and the extent of invasion of tissues was much less than in those infected by F-1.

DISCUSSION.—On the basis of these investigations, the difference in wilt-inducing potential between culture filtrates of a highly pathogenic and a weakly pathogenic strain of *P. solanacearum* can be attributed mainly to a heat-stable substance in the filtrate of the former. Although the exact chemical composition of this wilt-inducing material has not been determined, the high viscosity of aqueous solutions of the material, its slow rate of dialysis, and preliminary chemical tests indicate that it is a highly polymerized complex polysaccharide with glucose as the main sugar constituent. In an investigation of various bacterial polysaccharides, Cooper and Preston (2) had reported previously that *P. solanacearum* occasionally produced a fructosan in peptone-sucrose broth cultures.

Most bacteria form an extracellular slime layer that is either firm and organized as a capsule or loose and released into the culture medium (4, 5, 17). With few exceptions, the slime layer consists of simple or complex polysaccharides. According to Dubos (4) and Knaysi (17), all slime-producing bacteria also form smooth mucoid colonies on solid media and cause an increase in the viscosity of the medium when grown in liquid cultures. This ability to produce slime can be lost through mutation (1, 4). As a result of this loss, colony morphology of the mutant strains differs from that of the original type and viscosity of broth cultures of the mutants is lower than that of the slime-forming strains.

On the basis of the above observations, it can be postulated that the wilt-inducing material produced by the highly pathogenic strain of *P. solanacearum* is essentially the extracellular slime layer of this bacterium. This is supported by the fact that the reticulate pattern of the slime formed by the virulent strain in diseased tissues and on solid media is the same as that of the wilt-inducing substance obtained by alcoholic precipitation from culture filtrates of the virulent type. Since both the weakly pathogenic and the non-

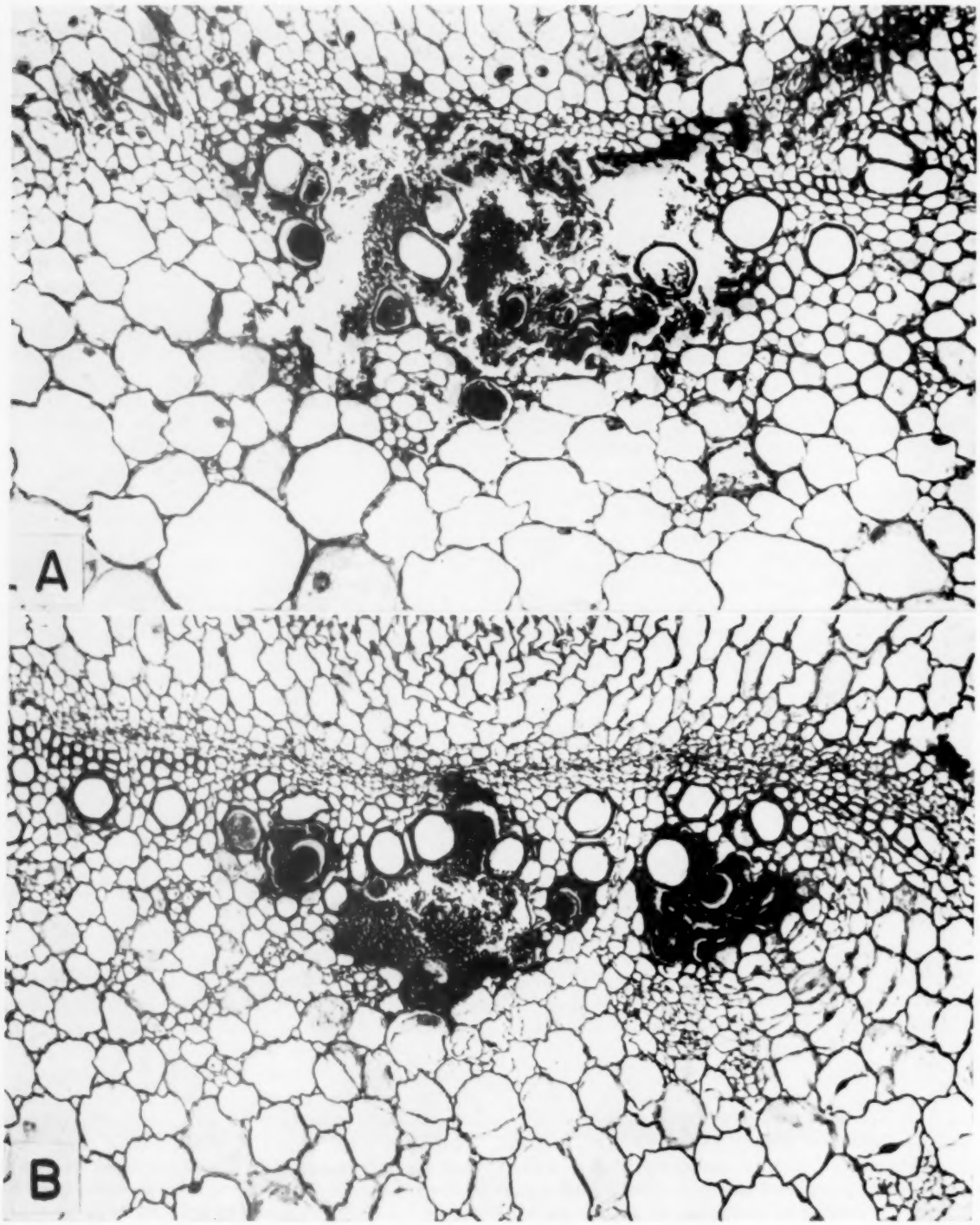

FIG. 3. Photomicrographs of transverse sections of the stems of tomato plants inoculated with 2 strains of *Pseudomonas solanacearum*. A) Highly pathogenic F-1 strain and B) weakly pathogenic B-2 strain.

pathogenic strains used in these studies originated from a single-cell culture of the highly pathogenic type (15), it also can be assumed that these strains are non-slime-forming mutant types. This assumption is supported by the demonstration in stained preparations that slime is not present around cells of the weakly pathogenic and nonpathogenic strains but present around cells of the virulent strains.

The experiments with tracheal fluid indicated that there was a wilt-inducing heat-stable substance in diseased plants. Furthermore, bacterial slime was present in the xylem tissue of diseased plants, as was demonstrated in stained preparations of bacterial exudates. It is recognized that this constitutes only indirect evidence that the slime material is the wilt-inducing factor in the tracheal fluid. Nevertheless, on the basis of the work completed in this study, the wilt-inducing slime fulfills most of the criteria of a vivotoxin as defined by Dimond and Waggoner (3).

In phytopathological literature there are relatively few instances of a demonstrated correlation between ability of a pathogen to form a specific injurious substance and its level of virulence, as has been shown for certain strains of the Granville wilt bacterium. It is significant that this relationship between slime production and virulence in *P. solanacearum* is analogous to that between capsule formation and virulence in certain strains of human and animal pathogens. In the classical research on *Diplococcus pneumoniae* Weichs. (Pneumococcus), it was demonstrated that smooth and mucoid colony types are encapsulated and virulent; in contrast, rough colony variants have lost the ability to produce a slime layer and are avirulent (4). Similarly, the formation of capsules has been correlated with virulence in *Klebsiella pneumoniae* (Schroet.) Trev. (18), *Hemophilus influenzae* (Lehm. & Neum.) Wins. (25), *Brucella* spp. (1, 10), and *Pasteurella pestis* (Lehm. & Neum.) Holl. (26).

In encapsulated human and animal pathogens, the capsular substance may protect the invading organisms against the action of phagocytes, antibodies, or the reticuloendothelial system of the host. Since there is a distinct similarity between the physiological effects of loose slime produced by nonencapsulated and that which is formed in a distinct layer around encapsulated bacteria (4), the slime of the Granville wilt bacterium, in addition to its role in wilt induction, may function in a manner similar to a capsule in its relationship to the bacterial cell and in the effects of its inherent chemical components. It is recognized that the functional protective value of a slime layer for a plant pathogen is quite different from that of an animal pathogen. It is possible, however, that the absence of slime around cells of the avirulent or weakly virulent strains exposes them to the effects of host substances that may restrict the development and multiplication of these strains following introduction into tomato or tobacco plants.

The difference in slime-producing ability between the highly pathogenic and weakly pathogenic strains can be related to the differences in colony morphology between these types as reported by Okabe (21, 22) and Kelman (15). The highly pathogenic type (F-1) produces slimy, moist, fluidal colonies; in contrast, the weakly virulent (B-2) or avirulent (B-1) strains are characterized by the small, butyrous, nonfluidal colonies typical of non-slime-forming variants.

The difference in pigmentation of the colonies on a tetrazolium chloride medium also can be explained on the basis of differences in slime production. Young fluidal colonies of the F-1 strain initially show only a faint pink coloration in the central areas; in contrast, the colonies of the B-1 and B-2 strains become deep red (15). On the basis of direct observations, the highly viscous slime layer has a low affinity for the reduced form of the tetrazolium salt. Thus, the pink color develops more slowly in the central areas of young colonies of the pathogenic strain and therefore the intensity of color remains lower than that which characterizes the non-slime-forming B-1 and B-2 colonies.

The present evidence fails to indicate that the Granville wilt bacterium causes wilting by the formation of plasma poisons or toxins as originally suggested by Hutchinson (13) and more recently by Kunz (19). In the experiments completed by both of these workers, toxins were isolated from cultures grown in beef bouillon, a medium that may itself cause injury to plants and one that favors the most rapid increase in populations of the weakly pathogenic variants, particularly in nonaerated cultures (16). Consequently the products obtained may not have been strictly those of the virulent strain. In the procedure followed by Kunz, the entire liquid culture medium containing bacteria that had grown for 2–5 months was used for alcoholic precipitation. This medium presumably contained products of autolysis of bacterial cells as well as high populations of the variant types. A toxic substance produced by *P. solanacearum* after 2–5 months of growth in culture may not play a role in nature where the course of the disease is very rapid and plants may be killed in 10–14 days. On this basis, it is possible to explain the differences between the properties and effects of the wilt-inducing substances obtained by Kunz and the wilt-inducing material obtained in the present investigation.

The ability to induce wilting in plants has been ascribed previously to extracellular polysaccharides formed by certain phytopathogenic fungi and bacteria. A wilt-inducing high-molecular-weight polysaccharide was obtained by Thomas (28) from culture filtrates of *Fusarium solani* f. *eumartii* (Carp.) Synd. & Hans. This material was considered to cause wilting by a mechanical blocking effect. Hodgson et al (9) studied the wilt-inducing properties of various polysaccharides of plant origin, of different high-molecular-weight substances such as carbowaxes, and of a glucosan formed by the crown gall bacterium, *Agrobacterium tumefaciens* (Smith & Towns.) Conn. From these studies it was concluded that for certain polysaccharides there was a direct relationship between molecular weight and ability to cause wilting and necrosis of leaflets

of tomato cuttings. Wilting was attributed mainly to a mechanical blocking of vessels. The polysaccharides that caused leaflet injury were those that formed clear aqueous solutions with relatively low viscosity. In general the approximate molecular weights of these materials were significantly lower than the molecular weights of those polysaccharides that formed opalescent or viscous solutions and caused a more generalized wilting with relatively little injury to leaflets. Recently, Leach et al (20) studied the wilt-inducing ability of a high-molecular-weight polysaccharide produced by *Xanthomonas phaseoli* (E. F. Sm.) Dows. This polysaccharide formed a very viscous opalescent aqueous solution and caused reversible wilting of cuttings of tomato, sunflowers, and beans. Leach et al concluded that wilting by this polysaccharide was caused by a mechanical blocking of the vascular tissues in the stem. With respect to general properties and effects on cuttings, the slime material of *P. solanacearum* is more similar to the polysaccharide of *X. phaseoli* than to the glucosan of *A. tumefaciens*. The roles of the polysaccharides of *X. phaseoli* and of *A. tumefaciens* in the mechanism of pathogenesis have not been determined, however, and neither of these pathogens causes wilting of plants under natural conditions.

On the basis of the present studies on the wilt-inducing slime of *P. solanacearum*, it is not possible to support the concept proposed by Grieve (7) and other workers that the primary cause of wilting is the mechanical plugging of vessels by masses of bacterial cells alone. Histological observations of completely wilted plants do not indicate that all or a majority of the vessels are entirely blocked by masses of bacterial cells, either in the stems or leaf petioles. Furthermore, plants inoculated with the weakly pathogenic non-slime-forming strain in several instances had vessels blocked by masses of bacteria, although these plants did not show wilting symptoms prior to the histological examination. It is possible, however, that the presence of large numbers of bacterial cells surrounded by a layer of viscous slime would enhance the blocking effect of the slime substance.

Evidence that the bacterial slime of *P. solanacearum* acts physically to impede water movement is supported by the observations on the wilt-inducing effects of other polysaccharides and also by the experimental results of van der Meer (29) and Grieve (7). Both of these workers showed that water movement was significantly slower in the stems of infected plants than in stems of healthy plants. Van der Meer also found that noninvaded wilted shoots recovered turgor when they were removed from diseased plants and placed in water. Furthermore, in attempts to obtain tracheal sap in this investigation, it was noted that it was extremely difficult to pull water through the stems of diseased plants by means of suction.

In a consideration of present information, it can be concluded that the slime produced by *P. solanacearum* plays a major role in the wilting of infected plants. It is suggested that the virulent strain of the bacterium, after it enters the host, multiples rapidly in the xylem tissue and forms slime in abundance. The slime causes a marked increase in viscosity of the vascular stream. As a result of the physical action of the slime and to a lesser extent of the bacterial cells, a gradual mechanical occlusion of the vessels occurs, and passage of water through the affected tissues gradually ceases.

It has been demonstrated that pectic and cellulolytic enzymes are produced by *P. solanacearum*, both in culture and in infected plants (11, 12). These enzymes are involved in the breakdown of tissues and may increase the severity of wilting as well as aid in the spread of the bacterium through the host. The weakly pathogenic or nonpathogenic strains of *P. solanacearum* do not produce the wilt-inducing slime, although these strains have the ability to form pectic and cellulolytic enzymes. Studies on the production of these enzymes and their role in pathogenesis will be discussed in a separate paper.

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PRODUCTION AND GERMINATION OF OOSPORES OF PHYTOPHTHORA INFESTANS[1]

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SUMMARY

Two sexually compatible mating groups were found in *Phytophthora infestans.* Oospores, within oogonia-bearing amphigynous antheridia, were formed in great abundance when any 1 isolate from 1 group of cultures of *P. infestans* was paired with any isolate from a second group on agar or on leaves of potato or tomato plants. Three isolates from Mexico, 26M, 42M, and 43M, composed 1 group, whereas 105 isolates from the United States, Canada, western Europe, South Africa, and the West Indies composed a second. One isolate from Mexico, 66M, reacted as did isolates in the second group. Oospores were not produced in abundance from intragroup pairings. Oospores were formed when compatible isolates were paired on liquid or agar media containing soybean meal, frozen Lima beans, V-8 juice, or ground cereals, or on steeped grain liquors. The mature oospores within oogonia varied from 24 to 56 μ in diameter, depending in part upon the isolate and the substrate used.

Oospores produced in paired cultures germinated in dung infusion and in potato-soil leachate preparations by producing germ tubes that usually bore terminal sporangia. These sporangia germinated by liberating zoospores or by forming germ tubes. The percentages of oospores that germinated in the above liquids ranged from 1 to 10. Many physical and chemical treatments failed to increase the germination percentage.

Two single-oospore isolates were obtained in pure culture. These were shown to be pathogenic to potato plants. One culture, derived from pairing a Mexican isolate of Race 1,2 with a United States isolate of Race 3, reacted as physiologic Race 0 when tested on differential potato hosts. The second oospore culture from pairing a Mexican isolate of Race 1,2 with an isolate of Race 1,4 from the United States reacted as did Race 1,4 but differed from it in growth type on Lima-bean agar. The mating reaction of both cultures was the same as that of the United States isolate used in the pairings.

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Sexual reproduction of *Phytophthora infestans* (Mont.) de By. has interested plant pathologists and mycologists since 1863, when de Bary first published proof of its pathogenicity to potato. This interest has continued because of the possible relationship of the sexual stage to the overwintering of the fungus and to the formation of new and more virulent races through genetic recombination.

Oospores of *P. infestans* were first reported by Smith (27) in 1875; however, examination of Smith's draw-

ings (1) indicates that he probably was observing oospores of closely related fungi. In 1890, Smorawski (29) described oogonia with paragynous antheridia in diseased potato tubers. In 1909, Jones (14) and Jones, Giddings, and Lutman (15) obtained oogonium-like bodies without antheridia in pure cultures on potato gelatin and on Lima-bean agar. Clinton (7) believed the culture medium used by them to be unsuitable to perfect the bodies further. The first report of oogonia with amphigynous antheridia and oospores in pure culture was made by Clinton (7) in 1911. They appeared when the fungus was cultured upon oat agar. He noted that the antheridia and oogonia originated upon different hyphae. Since Clinton's report, several investigators have observed oospores in cultures on oat agar and other media. Pethybridge (23) obtained oogonia without antheridia in oat agar. In 1913, Pethybridge and Murphy (25) reported an abundance of oospores produced by certain isolates on rolled-oat medium. A large number of oospore-bearing oogonia without antheridia were observed. Oospores thus produced were considered to be parthenogenetic. In 1914, Pethybridge (24) observed that the oogonial incept arises from a hypha near an antheridial hypha that develops first. The incept then penetrates the antheridium at its base, grows through it, and breaks out at the top, where it swells and produces the oogonium. Rosenbaum (26) found oospore-like bodies in one of several strains grown on oat agar; 95 per cent were without antheridia; the rest showed attachments that "might or might not have been antheridia." Lohnis (19) reported the occurrence of oospores within oogonia on raw potato tubers. De Bruyn (8, 9) found oospores in cultures on corn agar, oat agar, raw potato tubers, cereal straw, and possibly in soil cultures. In 1923, Eriksson (10) described oospores in potato leaf tissue at the periphery of primary lesions and observed their germination by germ tubes emerging through stomata. Berg (3) observed oogonia with amphigynous antheridia in cultures from Europe, Australia, and Holland but none in American cultures. In 1927, Murphy (21) found oogonia with parthenogenetic oospores; occasionally antheridia were present. Szymanek (30) discovered oogonium-like bodies in pure cultures in sterilized soil. Tucker (31) obtained oospores from old cultures grown on Lima-bean agar and on oatmeal agar. Following inoculations, Kotila (16) observed what she considered to be oospores in leaves and tubers of potato and in leaves of commercial tomato plants, as well as in *Lycium halimifolium* Mill., on *Solanum dulcamara* L., on *S. rostratum* Dunal, and on oat straw.

The first report of oospores occurring in interspecific crosses was made by Clinton (6) in 1911. He reported that oospores of the *P. infestans* type were more numerous when that species was paired in culture with either *P. phaseoli* Thaxt. or *P. cactorum* (Leb. & Cohn) Pethyb. than when it was grown alone. He considered the spores thus produced to be hybrids and assumed that *P. phaseoli* and *P. cactorum* supplied the antheridia that stimulated production of oospores. *P. phaseoli* and *P. cactorum* have since been considered to be homothallic (4, 17), and Leonian (17) suggested that *P. infestans* is also homothallic. Barrett (2), however, recently reported the occurrence of oospores in mixed cultures of *P. infestans* and *P. drechsleri* Tucker. A culture from a single germinated oospore from the cross apparently was established. This report (an abstract) is confusing because of a typographical error: the term "female *P. infestans*" appearing in the next to last sentence should read "female *P. drechsleri*."[4]

In earlier work with physiologic races of *P. infestans*, the authors became interested in the genetics of the fungus and particularly wished to study the inheritance of race characteristics. Since many different isolates had been accumulated from various parts of the world, it seemed logical to determine if any possessed the ability to produce oospores and, if so, to determine if they were homothallic or heterothallic. If heterothallic isolates were found, it might be possible to study the genetics of the fungus, provided the oospores could be induced to germinate. This paper reports the results of studies on the production and germination of oospores of *P. infestans*. Preliminary reports of these results have been given previously (13, 28).

MATERIALS AND METHODS.—Over a period of several years, 105 isolates representing 14 different potato physiologic races and 2 tomato races of *P. infestans* were obtained from potato- and tomato-growing areas of the United States, the British Isles, western Europe, Africa, Canada, Mexico, and the West Indies. Stock cultures were maintained on frozen-Lima-bean agar (blended frozen Lima beans, 150–250 g; agar, 15–20 g; distilled water to make 1 liter).

To determine if mating groups existed within these isolates, they were grown in mixed cultures of various combinations. Natural and synthetic liquid and solidified media, as well as potato and tomato leaves, were used. The medium used most extensively was soybean-meal agar (ground soybeans, 15 g; agar 20 g; distilled water to make 1 liter). Other media are listed specifically along with the experimental results. The media were seeded with either mycelial tufts or sporangial suspensions. The mixed cultures were grown at 18°–20°C.

Oospores produced in the mixed cultures were harvested by 1) the blending and centrifugation of agar media containing oospores, 2) the liquefaction of frozen-Lima-bean gelatin, and 3) the stripping of agar media from Petri dishes so as to leave oospores adhering to the glass bottoms. The last method was used almost exclusively for germination experiments.

The materials used in germination tests with oospores produced in mixed cultures included organic and inorganic chemicals, potato-soil leachate, and dung infusions. The potato-soil leachate was prepared by pouring distilled water on the soil around a large potted potato plant and then collecting at the bottom

[4] Personal correspondence with Dr. Barrett, November 27, 1956.

of the pot the first 100 ml that passed through the soil. The leachate was filtered through paper and then sterilized by autoclaving or by passage through a bacteriological filter. The dung infusions were prepared by steeping 40 g of horse, cow, sheep, rabbit, or guinea pig dung in 1 liter of distilled water. The dung was removed by filtering, and various dilutions of this stock solution were sterilized by autoclaving. Cheo and Leach (5) demonstrated that autoclaving did not destroy the principle in horse dung that stimulated germination of spores of *Ustilago striiformis* (West.) Niessl. The various organic and inorganic chemicals used in these studies are listed along with the experimental results.

Pathogenicity tests of germinating and nongerminating oospores were made by injecting potato plants with such oospores suspended in water or in horse-dung infusion or by spraying the foliage with oospore suspensions. Zoospore-sporangium suspensions from single-oospore cultures growing on Lima-bean agar were used to inoculate potato plants by spraying the foliage. Inoculated plants were incubated for 12–24 hours in either a dew chamber (20) or a moist chamber maintained at 18°–20°C.

EXPERIMENTAL RESULTS.—*Oospore production by single isolates.*—Preliminary experiments designed to determine if heterothallism exists in *P. infestans* were begun in August, 1955, at West Virginia University. Thirty isolates of their stock culture collection were grown in various combinations on soybean-meal agar seeded with sporangial suspensions.

Seven days after seeding, a few oogonia with amphigynous antheridia were observed in a number of the mixed cultures. These oogonia, which contained oospores, appeared in localized sections of a plate in numbers ranging from 10 to 200 per Petri dish and were golden brown when mature. Although antheridia were observed on many of the oogonia, they were not found on many others.

Since these first results suggested that mating groups may have been present, all isolates in mixtures that produced oospores were grown singly and in pairs. These tests revealed that a number of these isolates would form a few oospores when grown alone and that pairing or growth in mixtures had no effect upon the number and type of oospores produced. Later experiments showed that this phenomenon is common in a large number of isolates growing upon soybean-meal agar and that the number of oospores produced by an isolate is variable.

Mating groups and oospore formation.—Upon completion of the preliminary experiment above, the results were reported to Fort Detrick, where similar mixed-culture experiments were carried out. Four isolates from Mexico, designated 26M, 42M, 43M, and 66M,[5] were included in this experiment. Examination of the cultures 3–5 days later revealed oospores in great abundance whenever isolates 26M, 42M, or 43M were paired with any other isolate (Fig. 1, A). Further pairing tests conducted at Fort Detrick and at West Virginia University revealed abundant oospore production (several hundred times that produced by single-isolate cultures) whenever one of these 3 Mexican isolates was paired with any of 105 different isolates obtained from the United States, Canada, western Europe, South Africa, and the West Indies. Mexican isolate 66M reacted as did isolates from the United States. Thus, 2 mating groups were established in *P. infestans*, one composed of 3 of the 4 Mexican isolates and the second composed of all other isolates tested. No oospores have been observed in paired cultures of compatible isolates separated by dialysis membranes or in cultures to which sterile filtrates from cultures of compatible isolates had been added.

The sexual structures in paired cultures appeared similar to those described by Pethybridge and Murphy (25) as occurring in single cultures. The most characteristic morphological feature is the amphigynous antheridium that adheres tenaciously to the oogonium (Fig. 1, C). The oogonial incept, described by Pethybridge and Murphy as passing through the antheridium, is usually visible. The protoplasm of the young oogonium is finely granular and homogeneous (Fig. 1, D) but becomes filled with oil globules within 2–3 days. Eventually the oil globules become indistinct, the protoplasm pulls away from the pigmented wall of the oogonium, and a smooth thick-walled oospore develops (Fig. 1, C).

When compatible isolates were paired by transfer of mycelial tufts, oospore formation began very soon after the 2 advancing mycelia joined (usually within 3–5 days) and continued for 3–4 weeks. With this method of seeding, oospore development was concentrated in a region of several cm^2 across the center or toward 1 side of the Petri dish; often as many as 250 formed in the area of a single focal plane of a microscope field 1.5 mm in diameter. When the agar medium was seeded with a mixed sporangial suspension, oospores formed more slowly and were more sparsely distributed than when mycelial tufts were used. With both methods, oospores were formed throughout the depth of the medium, provided it did not exceed 3 mm.

Oospores formed in liquid, solidified gelatin, or agar media and in detached tomato and potato leaflets floated on water. In addition to soybean meal, other natural media used successfully in either liquid or solid form included 1) ground frozen Lima beans; 2) ground cereals including rye, oats, rice, sorghum, millet, wheat and barley[6]; 3) steeped grain liquors of the above cereals[7]; and 4) V-8 juice.[8] Oospores formed

[5] Obtained from J. S. Niederhauser, The Rockefeller Foundation, Mexico D. F., Mexico, via the U. S. Department of Agriculture.

[6] Fifteen g of ground grain per liter of distilled water.

[7] Prepared by steeping 250 g of grain per liter of distilled water.

[8] Two hundred ml of Campbell's V-8 juice (a blend of vegetable juices) and distilled water to make 1 liter. The reaction was adjusted to pH 5 by adding 3 g of $CaCO_3$ before filtering and autoclaving.

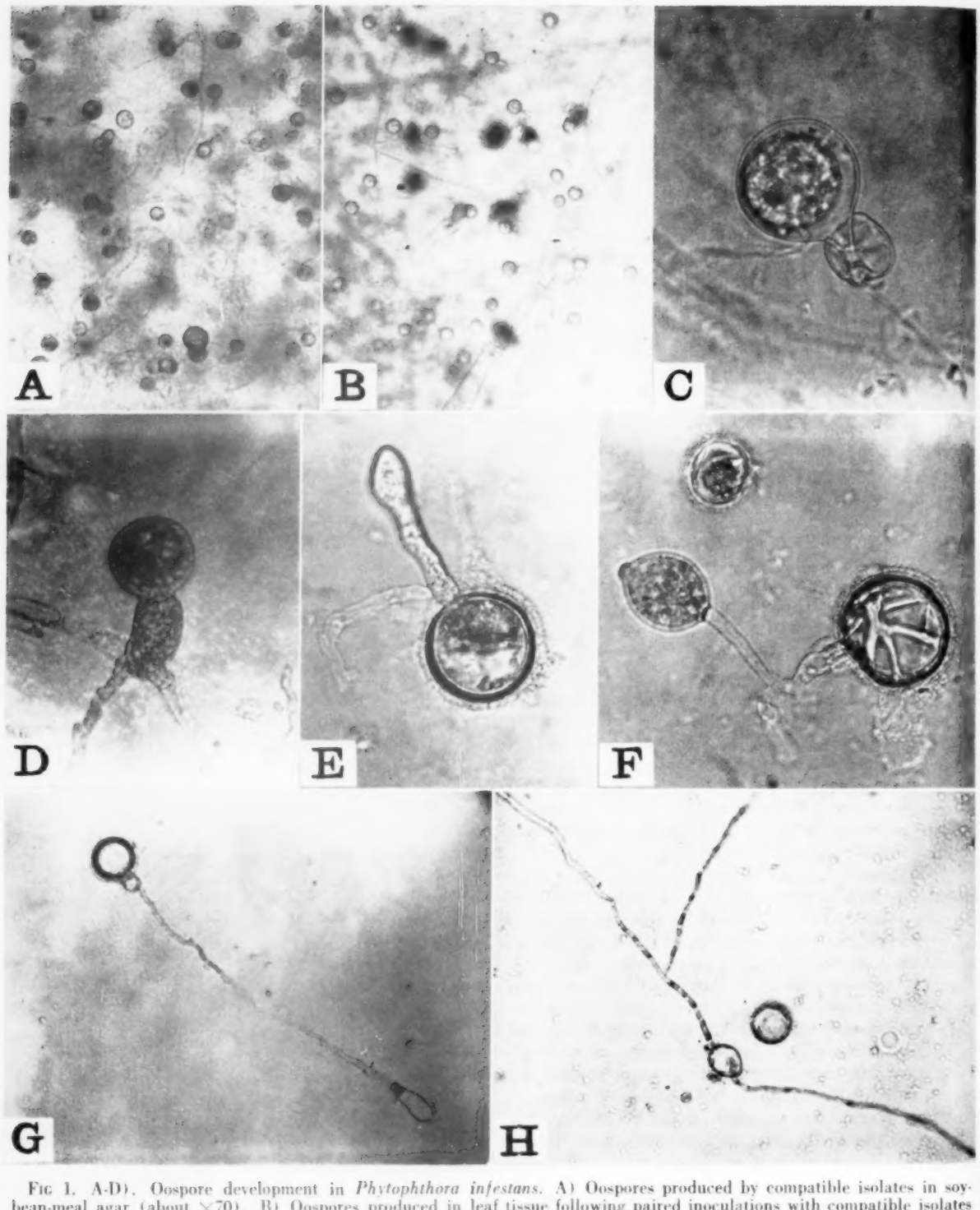

FIG 1. A-D). Oospore development in *Phytophthora infestans*. A) Oospores produced by compatible isolates in soybean-meal agar (about ×70). B) Oospores produced in leaf tissue following paired inoculations with compatible isolates (about ×70). C) Oogonium containing maturing oospore and showing the attached amphigynous antheridium with the oogonial incept (about ×500). D) Early stage in oospore development, showing hyphal attachments of the antheridium and oogonium (about ×350). E–H). Stages in oospore germination in *P. infestans*. E) Early stage of oospore germination. Note migration of protoplasm from oospore (about ×400). F) Germinated oospore with germ tube and terminal sporangium (about ×400). G) Germinated oospore with terminal sporangium that has liberated zoospores (about ×150). H) Germinated oospore showing direct germination of terminal sporangium (about ×150).

more rapidly in frozen-Lima-bean agar and in V-8 juice agar than in any other medium tested. This was presumably due to the vigorous rate of growth on these media. Several modifications of a glucose-asparagine synthetic medium (18) were tested but none was favorable for oospore formation, although limited mycelial growth occurred.

Oospores were formed in leaf tissue when adjacent inoculations with 2 compatible isolates were made on leaflets. The inoculations were made by placing filter paper disks saturated with sporangial suspensions on the leaflets; the inoculated leaves were then incubated in a dew chamber at 18°–20°C for 12–24 hours. Plants were then moved to a 21°C greenhouse, where lesions developed in 2–4 days. The leaflets with coalescing lesions were detached and floated on water in Petri dishes. Within 7 to 10 days after inoculation, microscopic observation showed oospores at the juncture of any 2 lesions formed by compatible isolates (Fig. 1, B). Since the developing oogonia in leaf tissue are quite similar in size and color to necrotic mesophyll cells, their presence is more discernible after the mesophyll cells have disintegrated almost completely than in the earlier stages of lesion development.

The color of the oogonia varies appreciably with the substrate, ranging from almost hyaline (in frozen-Lima-bean medium) or moderately brown (in soybean-meal medium) to a very dark brown, red brown, or orange brown (in millet and sorghum media) or a bright golden color (in steeped-barley medium). Oogonia from detached leaves are moderately pigmented and closely resemble those from soybean-meal medium. Mature oospores vary from 24 to 56 μ in diameter; the average being 30–40 μ, depending in part on the isolates and the substrate. Table 1 shows the variation in size of oospores from 1 pairing on various substrates. No correlation appeared between oospore size and oogonial pigmentation or between either of these factors and ability to germinate.

Oospore germination.—In April, 1956, germination of oospores produced in paired cultures of *P. infestans* was observed when dung infusions were used as the germinating liquid. Oospores usually germinated by the formation of germ tubes terminated by sporangia. Some of the tubes were shorter than the diameter of the oospore, whereas others were long and crooked and showed intercalary swellings. Many germ tubes branched and produced more than 1 terminal sporangium. Occasionally, the protoplasm withdrew from a branch without sporangial formation. Rarely, terminal sporangia were forced into a lateral position by continued growth of the germ tube. Although 1 or 2 germ tubes or branches of the same germ tube commonly emerged from the same opening in the oogonial wall, as many as 4 have been observed. Occasionally, the germ tubes of oospores continued to grow without producing sporangia.

Oospores begin germinating after 2–5 days in the germination medium. The spores continue to germinate a few at a time for several weeks. The most common point of emergence of the germ tube is through the oogonial stalk and the antheridium, apparently a point of weakness in the oogonial wall. Occasionally, germ tubes emerge directly through a rupture in the wall of the oogonium. Shortly after emergence, the tip of the germ tube begins to swell (Fig. 1, E). The protoplasm withdraws from the oospore and begins to flow into the swelling. About 24 to 48 hours after emergence, all of the protoplasm migrates into the swelling and a cross wall forms, delimiting a sporangium that remains attached to the germ tube (Fig. 1, F). These sporangia appear to germinate directly or indirectly in the same manner as those formed asexually. Thus, a sporangium may form a germ tube at 20°C (Fig. 1, H), whereas it may form zoospores at 12° (Fig. 1, G).

TABLE 1.—*Size of oospores of Phyphthora infestans resulting from pairing isolate WV4 with isolate 43M on different substrates*

Substrate	Size in μ[a]		
	Minimum	Average	Maximum
Potato leaves	24.5	31.5	38.5
Soybean-meal agar	28.0	41.0	56.0
Millet agar	31.5	40.6	52.5
Rye agar	31.5	40.9	49.0
Frozen-Lima-bean agar	27.0	34.1	42.0

[a] Based on the measurement of 200 mature oospores within oogonia.

Germination occurred in 1:10, 1:25, and 1:50 dilutions of the stock horse-dung infusion but was slightly better in the 1:25 dilution than in the other 2 concentrations. Little or no germination occurred, however, if oospores were pretreated with higher concentrations of the infusion. Similar concentrations of cow- or sheep-dung infusions induced germination equal to that occurring in horse-dung infusion.

Pretreatment with calcium hypochlorite solutions (10 per cent of saturation to saturation) for 24 hours before treatment with a 1:25 dilution of horse-dung infusion induced more rapid germination than did pretreatment for an equal period of time with distilled water. The total amount of germination over a period of several weeks, however, was not materially increased. Potato-soil leachate induced approximately the same amount of germination as did dung infusions. The effectiveness of the leachate was not altered by autoclaving for 20 minutes at 15 lb. steam pressure. Another preparation inducing oospore germination was a mixture of 9 vitamins: thiamine-HCl, riboflavin, pyridoxine-HCl, calcium pantothenate, *p*-aminobenzoic acid, nicotinic acid, choline chloride, inositol, and D-biotin.

Although the above treatments induced oospore germination, the percentage of spores that germinated was relatively low. At the optimum concentration of horse-dung infusion, a total of 3–4 per cent of the spores usually germinated. Germination did not exceed 1 per cent in many experiments but it approached 10 per cent in a few experiments. Much variation in percentage germination occurred among apparently identical treatments in different experiments, in differ-

ent replications of the same treatment, and in different areas of the same Petri dish. Such erratic germination made it difficult to evaluate the effectiveness of different germination treatments.

Occasionally, a single germinated oospore was observed in control plates (distilled water). More frequently, small numbers of oospores were observed to germinate *in situ* in old Petri dish cultures. In a few old V-8 juice cultures, a large number (approximately 50 per cent) of empty oospores have been observed, but it was difficult to determine if germ tubes were present on the empty spores.

A number of other treatments and pretreatments were studied, but none had any appreciable effect on oospore germination. Physical pretreatments followed by treatment in horse-dung infusion or in distilled water were used as follows: exposure to 40°C; drying; alternate wetting and drying; aging for 5–6 months in agar culture or under moist soil at 12° and −20°; alternate freezing and thawing (−20° to 20°) for several months; and aging for several months in a 20° dew chamber that periodically received potted potato plants. None of these treatments enhanced germination. Prolonged heat, drying, or aging decreased subsequent germination in horse-dung infusion.

Chemicals used unsuccessfully as treatments or pretreatments included the following: ethyl formate; ethylene chlorohydrin; naphthalene acetic acid; oxalic acid; 2,4,5-trichlorophenoxyacetic acid; isopropylacetate; potassium acetate; salicylaldehyde; sodium citrate; sodium oleate; sodium thiocyanate; pyridine; D-xylose; furfural; D-glucose; hypoxanthine; and the mineral salts, ammonium nitrate, anhydrous potassium carbonate, calcium carbonate, dipotassium phosphate, magnesium sulfate, potassium nitrate, monobasic sodium phosphate, and sodium sulfate in various combinations. Although many of the above compounds have been reported (12, 18) to stimulate the germination of spores of other fungi, none of them, when used without horse-dung infusion, elicited any greater oospore germination than did distilled water.

Because of the erratic germination, preliminary experiments involving pH or temperature were inconclusive; however, germination has been observed at temperatures ranging from 12° to 25°C.

Plant infection.—The pathogenicity of 2 cultures derived from single oospores has been tested with respect to sexual group and to physiologic race characteristics. Over 300 oospores that germinated under horse dung infusion or under potato-soil leachate were picked and transferred to frozen-Lima-bean agar or to potato tubers. Four of these continued to grow, and of these only 2 were obtained in pure culture. One culture derived from paired isolates of 43M (Race 1,2) and WV-4 (Race 1,4) reacted as Race 1,4 when tested upon a series of differential potato plants but differed from it in growth rate and type on Lima-bean agar. The second culture derived from isolates 42M (Race 1,2) and 62 (Race 3) behaved as physiologic Race 0, when tested upon varieties Cobbler (recessive), Essex (R_1), 3RC-8 (R_2), Pentland Ace (R_3), and DXD-3 (R_4). Both oospore cultures were sexually compatible with isolates of the Mexican group only. No infection has been obtained by atomizing oospores in suspension directly on plants, even when the plants remained under moist conditions for periods up to 2 weeks. Likewise, plants injected with oospores suspended in either water or horse-dung infusion failed to develop lesions after 2 weeks.

Discussion.—The results reported herein showed that the 3 Mexican isolates (26M, 42M, and 43M) were acting as 1 mating group and that the more than 100 isolates from various parts of the world including 1 from Mexico were acting as another mating group. It remains to be determined if all isolates formed both antheridia and oogonia or whether some isolates in each group are bisexual and others unisexual. The fact that many isolates produced a few perfectly formed oogonia with antheridia when grown alone on soybean-meal agar suggests that the isolates have both genes for sex but are highly self incompatible.

To determine if hybridization is occurring when isolates from the 2 groups are brought together, it will be desirable to establish single-oospore cultures and to determine if segregation for sex occurs in single-zoospore subcultures. Further work is being continued at West Virginia University with single-oospore cultures to determine if recombination will give rise to other races of *P. infestans*.

If it is found that hybridization and recombination occurs, it would be logical to assume that the sexual stage plays a part in development of physiological races as well as in survival of *P. infestans* in Mexico, since both mating groups were represented by the 4 isolates from Mexico. Niederhauser (22) has pointed out the importance of the discoveries reported here and has discussed their significance in the late blight problem in Mexico and elsewhere. At present it is believed that the sexual stage does not play a part in the disease cycle in the United States, in Canada, and in western European countries. This belief is based on the fact that so far only 1 of the mating groups has been found in isolates from these countries. At present, it is not known why only 1 mating group occurs in North America. Gallegly and Galindo (11) found that the 2 groups exist in Mexico in a ratio of approximately 1:1. It seems unlikely that only 1 group has been introduced into the United States with the complete exclusion of the other. Further pairing of isolates may yet demonstrate the existence of both mating groups in North America and Europe.

Since both mating groups were found in Mexico, it seems logical to assume that both groups occur also in other Central and South American countries where tuber-bearing species of *Solanum* grow undisturbed in nature and where the environment is similar to that of Mexico.

The fact that mating groups exist in *P. infestans* may be used to explain why earlier workers have failed to find oospores or found them in small numbers. It is likely that only 1 of the mating groups existed in the

areas where these workers collected isolates of the fungus. Since these earlier workers found oospores in relatively small numbers, it is believed that these spores were of the type described herein as occurring in certain single-isolate cultures.

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GROWTH INHIBITION AS A MECHANISM IN DUTCH ELM DISEASE THERAPY[1]

Carl H. Beckman

SUMMARY

Growth-promoting or growth-inhibiting action of 5 therapeutically active chemicals was determined in tests with pea-epicotyl sections and field-grown elms. These chemicals differed in their ability to promote growth, but showed a common ability to inhibit growth, particularly of sapwood, when normal concentrations of indoleacetic acid were present. A good correlation was found between chemically induced inhibition of development of springwood and reduced incidence of symptom expression in elms inoculated with *Ceratostomella ulmi* (Schwartz) Buis. Reinoculation following cessation of chemical treatment and resumption of normal springwood development resulted in the usual incidence of symptom development.

A clarification of the mechanisms of symptom induction and therapeutic action in wilt diseases would be of considerable aid in improving treatment of diseased plants.

Ronsdorf (17) and Beckman (2) have reported that the Dutch elm disease pathogen, *Ceratostomella ulmi* (Schwartz) Buis., produces auxin in culture. Several investigators have found that exoenzymes are produced by wilt pathogens (11, 23, 12, 2). These metabolites could effect a shift in the normal host metabolism to induce vascular occlusion and wilt.

The metabolic state of the host appears to have a pronounced effect on this host-pathogen interaction. Symptom development in elms, which are of discontinuous growth habit, is most severe when inoculation occurs during the period of rapid spring growth. Physiological as well as morphological factors appear to be responsible (1, 25). In tomato, which has a continuous growth habit, succulent rapidly growing plants produced under optimum moisture conditions are subject to Fusarium wilt, whereas woody slow-growing plants that result from low moisture are less affected (6).

Several lines of evidence indicate that therapeutants alter this metabolic state of the host to reduce susceptibility. Low fungitoxic value of many therapeutants and therapeutant-treated tissues and the necessity for preinoculation treatment appear to preclude a more direct action against the fungus or fungus metabolites. In addition, many therapeutic agents show formative effects on host plants, whereas known growth regulators often inhibit symptom expression (3, 7).

With these evidences pointing toward the importance of metabolic state of the host both for symptom induction by the pathogen and symptom inhibition by therapy, a study of the metabolic response of the host in relation to disease production and prevention has been undertaken. The effect of therapeutic agents on growth promotion and/or inhibition in elm trees and pea epicotyls is reported here.

MATERIALS AND METHODS.—A pea-epicotyl-section test was used to compare growth-regulating properties of several therapeutically active agents. These agents were sodium-2-benzothiazolylmercaptoacetate (8), referred to as BTMA, and the related compound sodium 4,5-dimethyl-2-thiazolylmercaptoacetate, referred to as 4,5-MTMA; 2,4-dichlorophenoxyacetic acid (7), referred to as 2,4-D; 4-dimethylamino-1,4,4a,5,5a,6,11, 12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1, 11-dioxo-2-naphthacenecarboxaminide (24), referred to as oxytetracycline; and polyethylenesorbitan monolaurate (24), a surfactant referred to as Tween 20.

Alaska peas were presoaked for 4 hours and then grown in quartz sand for 7–8 days in darkness at 23° ±1°C; cuttings were prepared according to the methods of Galston and Hand (10). Sections were held in a 1 per cent sucrose solution for 2 hours before treatment. Growth-regulating properties of the test chemicals under conditions of low auxin were determined by placing 10 epicotyl sections in a petri dish with 10 ml of 1 per cent sucrose solution containing either 5, 50, or 500 ppm of chemical. Growth-regulating properties under conditions of optimum auxin level were determined in portions of the above solutions to which 5 ppm of indoleacetic acid (IAA) had been added.

In subsequent field experiments, nursery-grown elms (*Ulmus americana* L.) 2–4 in. in diameter at breast height (d.b.h.) were used. Treatment was as follows. Two ¼-in. holes were bored horizontally and tangentially through the outer sapwood of the main stem from a single entrance hole approximately 6 in. above the base. A second set of such holes was bored from the opposite side of the main stem and approximately 6 in. above the first. These holes allowed treatment of the entire sapwood cylinder without girdling. Dry chemical (approximately 2 g/in. diameter) was packed into these holes. This chemical was generally found to be dissolved and taken into the sap stream during 8 hours of daylight. No noticeable change in the rate of uptake occurred following as many as 11 reapplications of chemical during a 6-week period.

Twenty-five trees were treated with a single application of 4,5-MTMA during the early bud-break stage of foliar development (May 2–16). Ten trees were given 3 weekly applications (May 2, 9, and 16). Growth effects on the variously treated trees and on 15 check trees were determined by periodic estimates of aver-

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age leaf length and measurements of current sapwood thickness in fresh twig sections. These trees were inoculated with *C. ulmi* through scalpel cuts into the sapwood of 3- to 4-year-old branches (June 5) when leaves had developed sufficiently to insure uptake of a drop of conidial suspension. Trees that showed no symptoms after 20 days were reinoculated. Symptom development was estimated visually as percentage of the crown showing foliar wilt.

RESULTS.—*Pea-epicotyl test.*—Pea-epicotyl sections

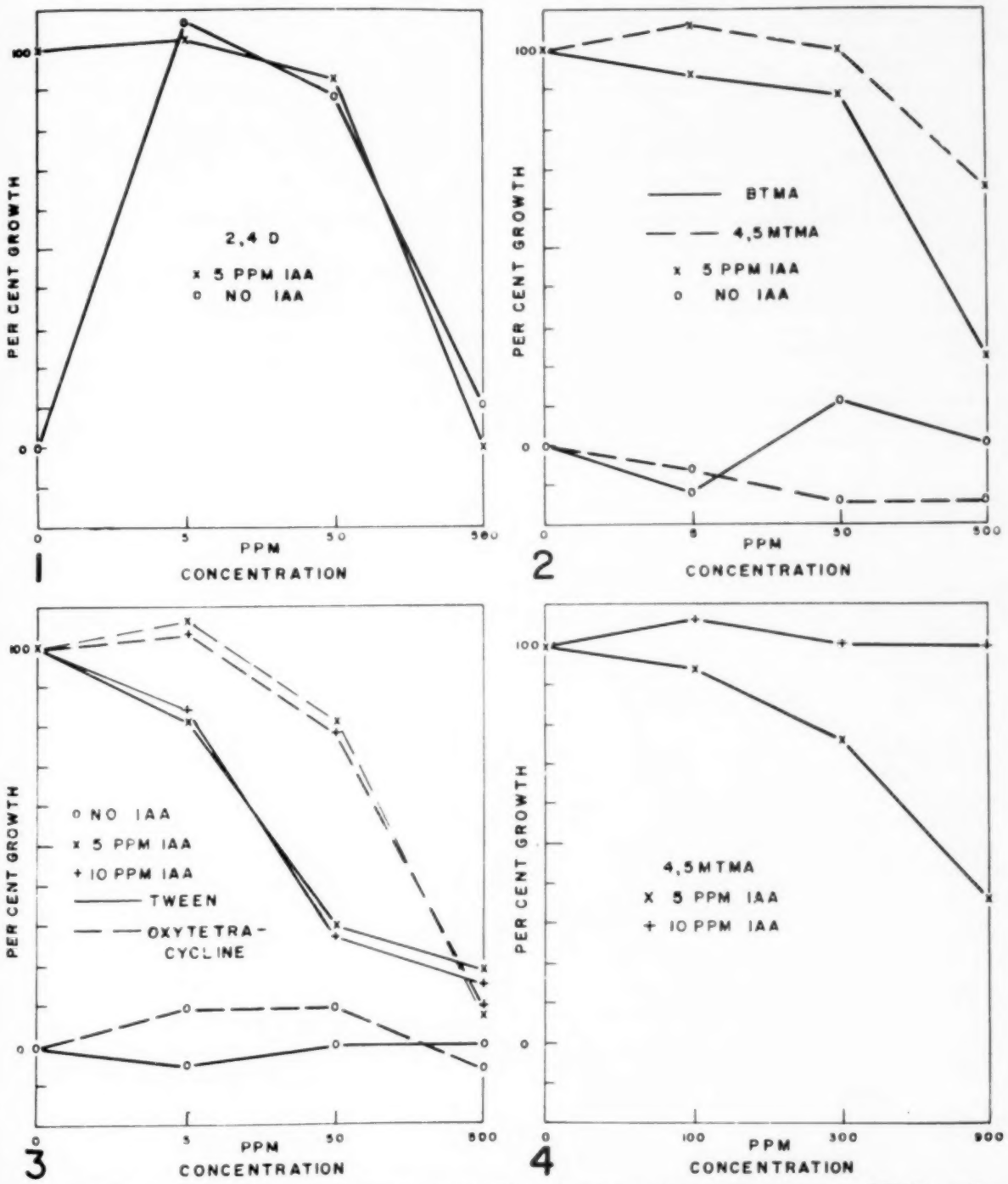

FIG. 1-4. The effect of 2,4-dichlorophenoxyacetic acid (2,4-D), sodium-2-benzothiazolylmercaptoacetate (BTMA), sodium 4,5-dimethyl-2-thiazolylmercaptoacetate (4,5-MTMA), oxytetracycline, and Tween 20 at various concentrations on the growth of etiolated pea-epicotyl sections treated with indoleacetic acid (IAA) at either 0, 5, or 10 ppm.

were placed in 1 per cent sucrose solutions containing 5, 50, or 500 ppm of test therapeutants to detect growth-regulating properties that might function in plants during periods of low auxin levels. Growth promotion comparable to that induced by indoleacetic acid resulted from treatment with 2,4-D. Little or no growth promotion resulted from treatments with BTMA, 4,5-MTMA, oxytetracycline, or Tween 20 (Fig. 1-3). Data were based on at least 2 replications of 20 epicotyl sections.

Growth-regulating properties of these chemicals that might function in plants during periods of optimum IAA levels were determined by incorporating 5 ppm IAA into portions of the above solutions. Growth or inhibition was calculated as a percentage of the difference between sucrose-solution checks and sucrose-plus-IAA solution checks. When concentrations of 5 ppm were used, growth of epicotyls in Tween 20 was 19 per cent less than that of the IAA check. Differences with other treatments were of doubtful significance. At the 50-ppm level, all chemicals except 4,5-MTMA and 2,4-D inhibited growth to various degrees, whereas at 500 ppm all chemicals inhibited growth considerably (Fig. 1-3).

Additional IAA (5 ppm) was added to portions of several of these solutions to determine if the inhibition could be reversed. This increase in IAA reduced the inhibition by 4,5-MTMA (Fig. 4) and by BTMA. It is apparent, therefore, that these compounds act competitively with IAA. In contrast, additional IAA did not relieve the inhibition by oxytetracycline or that by Tween 20. These compounds commonly act as enzyme inhibitors and as such may have reduced growth.

Elm test.—Since tissues as well as different plant species vary in their response to growth substances, the effects of treatment on leaf length and on xylem tissue development in field-grown elms were determined. The material 4,5-MTMA was used in this experiment, since both promotion and inhibition of growth resulted from various dosages of this chemical in the pea epicotyl test. Growth response and therapeutic activity at various dosages were compared, since this might indicate the mechanism of therapeutic action.

A moderate (30 per cent) inhibition of foliar expansion and a strong (70 per cent) inhibition of sapwood development was found 33 days after a single application (2 g/in. diameter) of 4,5-MTMA into 25 trees (Fig. 5). Three weekly applications into 10 trees resulted in an average of 65 per cent foliar and 77 per cent sapwood inhibition.

On the 34th day following initial treatment (June

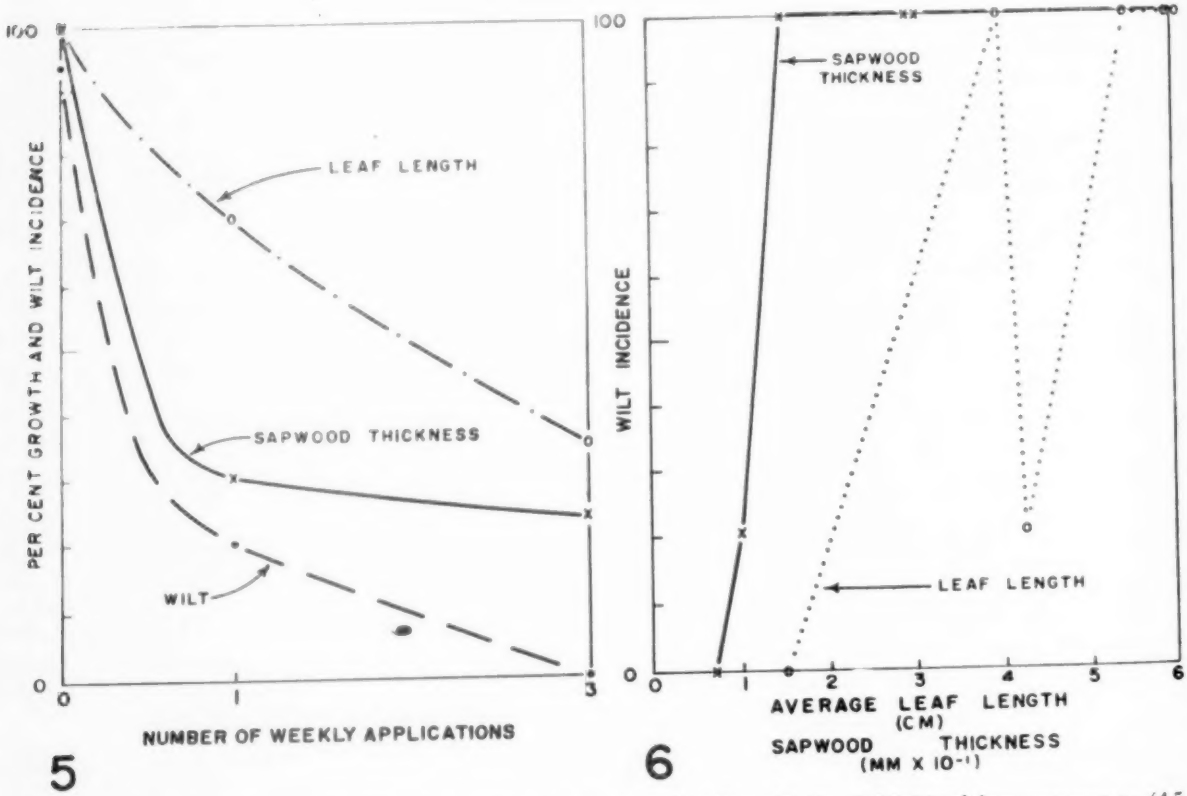

FIG. 5-6. FIG. 5. The effect of number of weekly applications of sodium 4,5-dimethyl-2-thiazolylmercaptoacetate (4,5-MTMA) in elms on length of leaves and thickness of sapwood at the time of inoculation and on incidence of wilt 20 days after inoculation with *Ceratostomella ulmi*.—FIG. 6. Size of leaf and thickness of sapwood at the time of inoculation or reinoculation with *C. ulmi* in relation to incidence of wilt 20 days after inoculation.

5), these and 15 check trees were inoculated with *C. ulmi*. Symptom incidence 20 days later was 0, 20, and 100 per cent in trees that had received 3, 1, and no applications of 4,5-MTMA, respectively (Fig. 5). These trees were reinoculated on June 25, which was 54 days after single, and 40 days after multiple, treatments were stopped. Normal sapwood growth had resumed at this time. This reinoculation resulted in 100 per cent infection. Leaf and sapwood development were determined again at the time of reinoculation. Integration of these and previously reported data (1) showed a good correlation between inhibition of sapwood development and inhibition of symptoms (Fig. 6). No such correlation was found between inhibition of foliar development and inhibition of symptoms. These results indicate a relationship between condition of the sapwood and susceptibility of the host.

Discussion.—The fact that elm trees are most susceptible to infection during the period of rapid spring growth suggests that the physicochemical condition of living cells of the host is a factor in host-pathogen interaction. Accumulated evidence indicates that therapeutic agents may increase disease resistance by altering this physicochemical state.

Results here indicate that 1 type of therapeutic activity is correlated with the inhibition of growth. Reports that active growth promoters, such as 2,4-D, also act as therapeutic agents appear, at first, incompatible with this conclusion. Any conclusions concerning such function, however, must take into account the concentration of the chemical applied, its transport, and the additive effect *in situ* of this chemical and the normal complement of auxin in the plant during the period of treatment. In the pea-epicotyl test, the activity potential of these chemicals was estimated. Growth promotion ranged from strong to none. When introduced with a physiologically active concentration of IAA, however, growth promoters and nonpromoters of growth alike showed little capacity to increase growth but instead showed a strong inhibiting activity.

When the factors of transport and normal auxin complement were introduced, as in the elm experiment with 4,5-MTMA, inhibition was again apparent, especially in the development of xylem tissue. A good correlation was found between this inhibition of xylem development and inhibition of symptom development following inoculation. After treatment was terminated and normal development of sapwood had resumed, reinoculation resulted in symptom development comparable to that in untreated trees. There was, then, a consistent association between inhibition of sapwood development and resistance to infection.

The relationship between resistance and inhibition of development of sapwood is not yet understood. At present, 2 possibilities are suggested. First, morphological differences may be responsible. Complete inhibition of springwood would necessitate inoculation into the previous summerwood, whereas intermediate inhibition could result in fewer, smaller vessels comparable to summerwood. The resistance of summerwood may in itself have a morphological basis (1). Secondly, this inhibition may result in fundamental changes in the physicochemical state of the host. As pointed out earlier, susceptibility of elm is low during the summer, whereas drought hardening reduces symptom development in tomato. Since in both circumstances differentiation would be great, and since growth and differentiation are held to be competitive processes (13), it is conceivable that many factors or agents that inhibit growth could increase differentiation (or maturation in the case of poorly differentiated tissue). Resistance of fiber cells to decay is dependent upon the degree of differentiation (13). Resistance to the action of enzymes of wilt pathogens, and therefore inhibition of symptom development, may also depend upon strong differentiation. If so, it is necessary to consider the tissues involved.

There is considerable evidence that a primary factor in wilting is the occlusion of vessels by gums and tyloses (4, 9, 18, 25). Chattaway (5) has shown further that gums and tyloses (in heartwood formation) arise from ray parenchyma cells that lie adjacent to, and are in intimate contact with, a vessel because of a common pit pair. A similar situation has been reported for pathological vessel occlusion in oak wilt (19). The physicochemical state of the ray-parenchyma cells may therefore be of primary importance in wilt development and therapy. Preston (15) describes the primary cell wall as a metabolically active portion of the cell that integrates with the cell protoplast. Thimann and Samuel (20) have shown that mechanical coherence in the cell wall is decreased, whereas permeability is increased, during growth. Auxins further condition a dissociation and expansion of cytoplasmic proteins (14, 22). The number of available sulfhydryl groups is increased (14, 16). These reports suggest that constituents of the entire cell, including the primary wall, are in a highly distended, permeable, and reactive state when under the influence of optimum auxin concentrations. These physiological changes can occur whether actual growth is possible or not (21). It is conceivable, therefore, that exoenzymes, which are known to be produced by many wilt-inducing organisms, may better permeate and have greater effect in cells that are in this highly permeable and reactive condition than in cells that are strongly differentiated. Such a hypothesis could account for greater symptom induction during periods of rapid succulent growth than during slow growth. Conversely, inhibitors that antagonize host- or pathogen-produced auxin, or that otherwise induce a more condensed, less permeable, and less highly reactive primary cell wall and protoplast than is normal, may reduce or prevent pathogenic enzyme penetration and catalytic activity.

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The Effect of Water on the Partial Sterilization of Barley Seed by Propylene Oxide and by Heat.[1] L. E. TYNER.[2] In studies on the relative resistance of the microflora of seed to sterilizing agents, it was found that partial elimination of the flora on the seed of Glacier barley was achieved by treatment with propylene oxide or with heat. In the first method, 0, 10, 20, or 30 per cent by weight of water was added to 50-g quantities of seed in 1-liter flasks. Propylene oxide in various amounts—0, 0.5, 0.75, 1.0, or 1.25 ml—was placed in the flasks, which were then sealed for 24 hours. The kernels were plated on potato-dextrose agar, and the colonies of bacteria and certain fungi that arose from them were recorded (Table 1). Bacterial colonies were present on almost all seeds following all treatments, although undoubtedly certain susceptible species were destroyed.[3] Various genera of fungi were present, but the incidence of only 4 was recorded. Three of the 4 genera of fungi were eliminated satisfactorily by propylene oxide from either dry or moistened seed; however, *Rhizopus* spp., which are annoying "weeds" in plating studies, were not

[1] Contribution No. 1639 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

[2] Officer in Charge, Edmonton.

[3] Ark, P. A. 1947. Disinfecting power of propylene oxide and propylene chloride in relation to phytopathogenic bacteria and fungi. (Abstr.) Phytopathology 37: 842.

TABLE 1.—*Fungal colonies[a] arising from 40 barley seeds that were moistened with water and sealed for 24 hours in flasks containing propylene oxide*

Water added %	Propylene oxide ml/l	No. of colonies[b] *Rhizopus*[b] spp.	*Fusarium* spp.	*Helminthosporium* and *Alternaria* spp
0	0	P	9	8
	0.5	P	0	0
	0.75	P	0	0
	1.0	P	0	0
	1.25	P	0	0
10	0	P	10	7
	0.5	0	0	1
	0.75	0	0	0
	1.0	0	0	0
	1.25	0	0	0
20	0	P	10	5
	0.5	0	0	0
	0.75	0	0	0
	1.0	0	0	0
	1.25	0	0	0
30	0	P	18	12
	0.5	0	0	0
	0.75	0	0	0
	1.0	0	0	0
	1.25	0	0	0

[a] Bacterial colonies arose from most seeds.

[b] P indicates that colonies were present in all plates but were not counted. Numerals indicate the number of colonies that arose from 40 seeds.

TABLE 2.—*Bacterial and fungal colonies from 40 barley seeds after addition of water and incubation for 24 hours at various temperatures*

Temperature °C	Water added %	No. of colonies: Bacteria Yellow	Bacteria Miscellaneous	*Rhizopus* spp.	*Fusarium* spp.	*Helminthosporium* and *Alternaria* spp.
40	0	40		4[a]	11	10
	10	40		5	6	5
	20	40		5	1	1
	30	40		4	0	1
45	0	32		5	10	8
	10	40		5	0	1
	20	20	20	3	0	0
	30		40	2	0	0
50	0	40		5	3	7
	10	40		5	2	1
	20		40	4	2	1
	30		40	1	0	0
55	0	0	40	5	2	4
	10	1	26	1	0	0
	20	1	36	0	0	0
	30	1	36	0	0	0
60	0	0	40	5	8	8
	10	8	32	3	0	0
	20	1	38	0	0	0
	30	0	40	2	0	0

[a] Number of plates infested. Eight seeds were plated in each of 5 plates. The total number of bacteria and fungi arising from each seed was recorded except in the case of *Rhizopus* spp. The rapid growth of this fungus made it possible to record only whether or not it was present in the plate.

eliminated from dry seed by any of the concentrations of propylene oxide employed. This fungus was completely suppressed by the chemical if water was added to the seed before addition of the propylene oxide. It appears that the gas penetrates moistened fungal or plant tissue more effectively than it does dry tissue.

In the heat treatments, water was added as above, and the seed was placed in cotton-plugged 200-ml flasks in a constant-temperature water bath at 40°, 45°, 50°, 55°, or 60°C for 24 hours. The temperature in the center of the mass of seed was about 4° less than that of the bath. The seed was plated on potato-dextrose agar immediately after treatment, and data were secured on the colonies of bacteria and certain fungi that arose from the seed (Table 2). Bacterial colonies arose from nearly all seed in each treatment. No attempt to classify them was made, but species of *Xanthomonas*, which were dominant at the lower temperatures, were suppressed at 55° or 60°. Control of fungi was not effected by any heat treatment of the dry seed, but the addition of moisture greatly facilitated their elimination, probably as a result of more efficient conduction of heat through moist seed. *Rhizopus* spp. were the most difficult to control, but at 55° or 60° they were fairly successfully eliminated from seed to which 20 or 30 per cent water had been added. —Plant Pathology Laboratory, Science Service, Edmonton, Alberta.

Internal Uredial Sori of Puccinia carthami with Reversed Polarity.[1] M. L. SCHUSTER.[2] The internal sporulation of rust fungi has been reported to include the production of 4 different spore forms in a variety of hosts. Adams[3] reported the production of internal uredia in the leaves of *Dianthus caryophyllus*, but here, as in other cases of internal sporulation, it has been difficult to determine from the illustrations whether or not the spores were borne in typical sori produced internally. Colley[4] illustrated his report of internal uredia and telia of *Cronartium ribicola* Fisher with photographs indicating that here typically formed sori were produced within the host. He also noted that such sori could be formed in an inverted position,

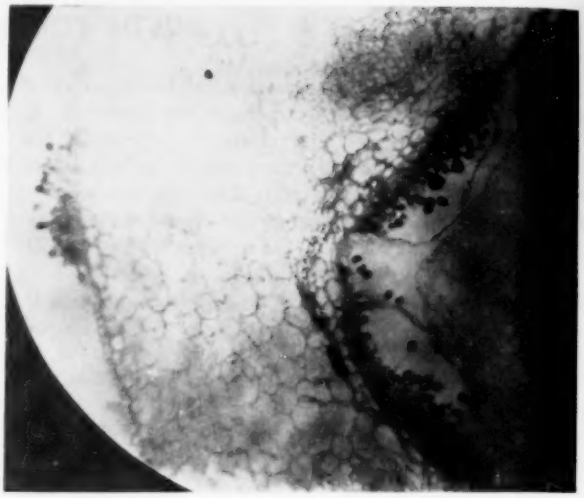

FIG. 1. Internal pustule of *Puccinia carthami* in hypocotyl of safflower. Note the extensive development of mycelium and the orientation of the internal pustule. The surface-borne sorus is located on the same side of the hypocotyl.

facing the center rather than the surface of the stems or petioles containing them. Schuster and Christiansen[5] earlier reported internal sporulation of *Puccinia carthami* Cda. within the hypocotyls and epicotyls of safflower, *Carthamus tinctorius* L. In their report it was not possible to distinguish the sorus, but the urediospore and teliospore production found within cavities of the cortex was oriented in a fashion that the apices of the internal sori were indistinguishable.

The purpose of this note is to report and illustrate the internal production by *P. carthami* of typical uredial sori oriented toward the center of the stem in cavities between the cortex and the stele (Fig. 1). The mycelium giving rise to the pustules was found within the cortex, but did not appear present in the stele. In these cases, there appears to be a reversed polarity that directs the apex of the uredia toward the central axis of the host. On the outside of the same portion of the stem, normal uredial sori were also produced externally. Presumably the clearly interpretable structure of the uredial sorus would have been less easily seen and identified if spore production had been long continued within the cavity occupied by the developing pustules.—Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska.

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[2] Associate Plant Pathologist, Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska.

[3] Adams, J. F., 1916. Internal uredia. Mycologia 8: 181-182.

[4] Colley, R. H., 1917. Discovery of internal telia produced by a species of Cronartium. Agr. Research 8: 329-332.

[5] Schuster, M. L., and D. W. Christiansen, 1952. A foot and root disease of safflower caused by Puccinia carthami Cda. Phytopathology 42: 211-212.